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Direct ^{99m}Tc Labeling of Pegylated Liposomal Doxorubicin (Doxil®) for Pharmacokinetic and Non-invasive Imaging Studies

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Nonstandard abbreviations: BMEDA: *N*,*N*-bis(2-mercaptoethyl)-*N'*,*N'*-diethyl-ethylenediamine; GSH: glutathione; GH: glucoheptonate; HMPAO: hexamethyl propyleneamine oxime; MPEG-DSPE: *N*-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt; HSPC: fully hydrogenated soy phosphatidylcholine; PET: positron emission tomography.

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ABSTRACT

Pharmacokinetic and organ distribution studies of liposomal drugs in humans are a challenge. A direct labeling method using ^{99m}Tc-*N*,*N*-bis(2-mercaptoethyl)-*N'*,*N'*-diethyl-ethylenediamine (BMEDA) complex to label the commercially available pegylated liposomal doxorubicin, Doxil® is introduced. Biodistributions of ^{99m}Tc-Doxil® in normal rats were performed to evaluate the feasibility of using it for monitoring the pharmacokinetics of liposomes encapsulating drugs. Labeling efficiency of ^{99m}Tc-Doxil® was 70.6 ± 0.8 % (n = 3). *In vitro* incubation of ^{99m}Tc-Doxil® in 50% fetal bovine serum (FBS) or 50 % human serum at 37^{o}C showed good labeling stability with 72.3 ± 3.6 % or 78.6 ± 1.8 % of activity associated with Doxil® at 24 h respectively (n = 3). There was a two-phase blood clearance with half clearance times of 2.2 h and 26.2 h after bolus intravenous injection in normal rats. Distribution of ^{99m}Tc-Doxil® at 44 h after injection had 19.8 ± 1.3 % of injected dose in blood, 14.1 ± 1.7 % in liver, 2.6 ± 0.3 % in spleen, 9.0 ± 0.8 % in bone with marrow, 6.0 ± 0.5 % in skin and 15.3 ± 4.3 % in bowel (n = 5). Unencapsulated ^{99m}Tc-BMEDA had a very rapid blood clearance with a half clearance time of only 0.12 h (n = 4). By using this ^{99m}Tc labeling method, biodistribution and pharmacokinetics of ammonium gradient liposomes encapsulating drugs can be determined by non-invasive scintigraphic imaging. This labeling method may be extended to ^{186}Re and ^{188}Re labeling to combine chemotherapy and radionuclide therapy for tumor treatment.

Proton (pH) or ammonium gradient liposomes are promising drug carriers because these liposome formulations can achieve high drug encapsulation capacity and high drug entrapment efficiency (Mayer et al., 1986a; Mayer et al., 1990; Haran et al., 1993). Pegylated liposomes have prolonged residence time in blood resulting in greater treatment effectiveness and lower treatment complications (Gabizon et al., 2003). Doxil®, a commercially available pegylated liposomal doxorubicin, uses an ammonium gradient to load and carry doxorubicin for clinical tumor treatment (Gabizon et al., 2003). Animal and human subject studies have been performed with Doxil® to treat various kinds of tumors (Gabizon, 1992; Harrington et al., 2000b). Because it is necessary to know the *in vivo* behavior of the therapeutic drugs trapped in liposomes as well as the free drugs after liposomal therapeutic drug administration, the pharmacokinetic study of liposomal therapeutic drugs tends to be more complex than routine drugs (Harashima et al., 2002).

Several gamma-emitting radionuclides can be used to label liposomes for monitoring the *in vivo* behavior of liposomes non-invasively (Harrington et al., 2000a; Harrington et al., 2000b). By using imaging systems, such as a gamma camera or a positron emission tomography (PET) camera, the whole body distribution of a radiolabeled carrier or a radiolabeled compound in each organ or tissue can be measured at different times in a live animal or in a human subject. By using non-invasive imaging, fewer animals are required to study the pharmacokinetics of drugs, making it more efficient and usually cheaper to perform the studies (Contag, 2002). In addition, increased statistical significance can be reached with fewer animals, because the images of each animal acquired at the initial time point can serve as the internal control at subsequent time points. Positron emission radionuclides, such as carbon-11

(^{11}C), nitrogen-13 (^{13}N) and fluorine-18 (^{18}F), may be used to label drug or carrier molecules (Aboagye et al., 2001). The disadvantage of these PET radionuclides is that they have relatively short half lives (^{11}C, 20.4 minutes; ^{13}N, 10.0 minutes; ^{18}F, 109.8 minutes), which makes it harder to trace the *in vivo* behavior of a drug or a carrier for long time periods.

Another group of radionuclides are single photon emitters, such as technetium-99m (^{99m}Tc) and indium-111 (^{111}In). These single photon emission radionuclides have longer half lives (^{99m}Tc: 6.007 h; ^{111}In: 2.80 d) permitting the monitoring of the *in vivo* distributions of ^{99m}Tc and ^{111}In labeled compounds for longer period using a gamma camera, which is readily available equipment in a nuclear medicine department. Liposome radiolabeling methods with ^{99m}Tc, ^{111}In or ^{67}Ga have been described (Phillips et al., 1992; Laverman et al., 1999; Essien and Hwang, 1988; Kassis and Taube, 1987; Woodle, 1993). These labeling processes attach radioisotopes to the surface of a liposome or trap radioisotopes in the liposome inner space. For surface labeling, a coordinate ligand needs to be attached to lipid or cholesterol molecules so as to bind the radioisotopes to a liposome by forming complexes with those metallic radioisotopes. Use of these surface labeling methods makes the radiolabeled liposomes structurally different from the actual liposomal therapeutic drugs used clinically. These surface modifications can potentially change the *in vivo* behavior of the liposomes and it may be argued as to whether these surface-altered liposomes can represent the clinically used liposomal therapeutic drugs.

In labeling radioisotopes to the inner space of a liposome by using a remote loading technique, a chemical gradient between the inner space and outer space needs to be constructed to induce high efficiency encapsulation and stable entrapment. One of the most frequently used ^{99m}Tc-liposome labeling methods applied to liposome studies uses glutathione (GSH) pre-encapsulated liposomes and ^{99m}Tc-hexamethyl propyleneamine oxime (HMPAO) (Phillips et al., 1992; Tilcock, 1999). To study the *in vivo* behavior of pH or ammonium gradient liposomes trapping drug molecules, an ideal approach would be the labeling of radioisotopes directly into the inner space of liposomes via pH or ammonium gradient mechanism. No previous study has been reported by using a direct labeling method to label pH or ammonium gradient liposomes with ^{99m}Tc. In this paper, we report a direct ^{99m}Tc labeling method to label a commercially available pegylated liposomal doxorubicin, Doxil®, using ^{99m}Tc- *N*,*N*-bis(2-mercaptoethyl)-*N'*,*N'*-diethyl-ethylenediamine (BMEDA) complex (Fig. 1). The *in vitro* labeling stability of ^{99m}Tc-Doxil® using this labeling method was studied by incubating it in 50 % serum at 37 ^{o}C. The normal rat distribution of ^{99m}Tc-Doxil® was also studied to assess the potential of using ^{99m}Tc labeling to study the pharmacokinetics and organ distribution of liposomes encapsulating drug molecules.

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Materials and Methods

Materials. Doxil®, a commercial product of ALZA Corporation and manufactured by Ben Venue Laboratories, Inc. (Bedford, OH), was purchased from University Hospital pharmacy (San Antonio, TX). Doxil® has an average particle size of 87.3 ± 8.5 nm before radiolabeling determined using 488 nm laser light scattering (Dynamic Light Scattering, Brookhaven Instruments, Holtsville, NY). It contains 2 mg/ml of doxorubicin, 3.19 mg/ml of *N*-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt (MPEG-DSPE), 9.58 mg/ml of fully hydrogenated soy phosphatidylcholine (HSPC) and 3.19 mg/ml of cholesterol. *N,N*-bis(2-mercaptoethyl)-*N',N'*-diethyl-ethylenediamine (BMEDA) was synthesized from a modification of the method by Corbin et al and the chemical structures were verified with ^{1}H and ^{13}C-NMR (Corbin et al., 1984). ^{99m}Tc-pertechnetate was purchased from Amersham Health Nuclear Pharmacy, San Antonio, TX. Glucoheptonate (GH) (Sigma, St Louis, MO) was used as an intermediate ligand to make ^{99m}Tc-BMEDA. Stannous chloride (Aldrich, Milwaukee, WI) was used as the reductant to make ^{99m}Tc-GH. Fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY). Human blood was donated by a volunteer. After the fresh human blood was centrifuged at 4000 rpm for 10 min, the serum was removed to a separate tube and aliquoted for *in vitro* stability study. Bio-Gel A-15m gel was purchased from Bio-Rad (Hercules, CA).

^{99m}Tc-Doxil® Preparation. A two-step method was used to prepare ^{99m}Tc-BMEDA. In the first step, ^{99m}Tc-sodium pertechnetate was reduced by stannous chloride and the reduced ^{99m}Tc(V) formed a complex with GH. In the second step, BMEDA was added

to ^{99m}Tc-GH solution and ^{99m}Tc-BMEDA was formed via ligand exchange mechanism.

The preparation of ^{99m}Tc-GH and ^{99m}Tc-BMEDA was as described before with minor modification (Bao et al., 2003a). In brief, ^{99m}Tc-GH was prepared in-house by pipetting 1.0 ml of freshly made degassed GH (10 mg/ml) solution (pH 7.0) containing 0.16 mg/ml $SnCl_2$ into a vial. Then, ^{99m}Tc-sodium pertechnetate (45 mCi (1.665 GBq)) in 0.50 ml of saline was added. The mixture was stirred at 25^{o}C for 20 min. The labeling efficiency of the ^{99m}Tc-GH was checked by paper chromatography eluted in methanol and paper chromatography eluted in saline.

BMEDA (3.5 µl (~3.9 mg)) was moved to a new vial. Then, 5.0 ml of degassed water and 4 drops of 0.05 M NaOH was added. The solution was stirred at 25°C for 40 min. After preparation, the BMEDA solution was labeled with ^{99m}Tc by adding 1.0 ml of BMEDA solution to 0.50 ml of ^{99m}Tc-GH (15 mCi (555 MBq)). After adjusting the pH to 8.0, the mixture was stirred at 25°C for 25 min. The labeling efficiency of the ^{99m}Tc-BMEDA was determined using paper chromatography eluted in methanol and paper chromatography eluted in saline. The labeling efficiency for ^{99m}Tc-BMEDA was greater than 85%. The average particle size of Doxil® after ^{99m}Tc labeling was measured using 488 nm laser light scattering (Dynamic Light Scattering, Brookhaven Instruments, Holtsville, NY). The resultant ^{99m}Tc-BMEDA was used for liposome labeling without further purification.

The ^{99m}Tc labeling protocol of Doxil® for labeling efficiency and *in vitro* stability study is as follows. Doxil® (0.5 ml) was added to the ^{99m}Tc-BMEDA (15 mCi (555

MBq)) solution prepared as above. The mixture was vortexed for 1 min and kept at 25^{o}C for 1 h. The ^{99m}Tc-Doxil® was separated from free ^{99m}Tc-BMEDA using Sephadex G-25 column chromatography eluted with PBS buffer, pH 7.4. The red color of Doxil® was used to visually monitor the collection of the ^{99m}Tc-Doxil®. The labeling efficiency was calculated by using the activity in Doxil® after separation divided by the total activity before separation.

The ^{99m}Tc labeling of Doxil® for the rat distribution study was similar to the procedure described above except 6 ml of Doxil® was labeled with 10 mCi (370 MBq) of ^{99m}Tc-BMEDA to meet the amount of Doxil® needed for injection at the clinical dosage. The labeled Doxil® solution was aliquoted into four parts and four Sephadex G-25 columns were used to separate each aliquot to remove the free ^{99m}Tc-BMEDA. The four eluted ^{99m}Tc-Doxil® aliquots were pooled and used for the rat distribution study.

^{99m}Tc-Doxil® *In Vitro* Labeling Stability. An aliquot of ^{99m}Tc-Doxil® after column chromatography separation was added to an aliquot of fetal bovine serum (FBS) (GIBCO, Grand Island, NY) or human serum in a 1:1 volume ratio and incubated at 37^{o}C. The amount of ^{99m}Tc activity associated with Doxil® at different times was measured using Bio-Gel A-15m Gel (Bio-Rad, Hercules, CA) spin column (Chonn et al., 1991). Bio-Gel A-15m gel (2.0 ml) was packed in a micro-column (Bio-Rad, Hercules, CA) by centrifugation at 1000 rpm for 2 min. Then, ten column volumes of PBS buffer, pH 7.4, were used to remove any ethanol and equilibrate column. At various times of incubation, 50 μl of ^{99m}Tc-Doxil® serum solution was added to an equilibrated spin column, the column was centrifuged at 1000 rpm for 1 min and the

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first fraction was collected in a tube. Then, 100 µl of PBS buffer, pH 7.4, was added to the column and centrifuged at 1000 rpm for 1 min and the second fraction was collected in a new tube. The elution process using 100 µl of PBS buffer, pH 7.4, was repeated 19 times and each fraction was collected after the centrifugation. The ^{99m}Tc activity in each fraction was counted using a Minaxiγ A5550 gamma counter (Packard, Downers Grove, IL). The percent ^{99m}Tc activity associated with Doxil® was calculated by summing the total ^{99m}Tc activity in the first six fractions divided by the total ^{99m}Tc activity of all 20 fractions. The relative doxorubicin concentration in each fraction was measured spectrophotometrically by mixing an aliquot (50 µl) of each fraction with 700 µl of methanol (EM Science, Gibbstown, NY) in a 1 ml cuvette. The absorbance was measured at 450 nm (Iden and Allen, 2001). The protein concentration in each fraction was determined using Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL). The remaining ^{99m}Tc activities in the spin columns after 20 fractions were less than 5%.

Normal Rat Distribution of ^{99m}Tc-Doxil®. To directly compare ^{99m}Tc-Doxil® with ^{99m}Tc-BMEDA, normal rat distributions were performed simultaneously. The animal experiments were performed according to the NIH Animal Use and Care Guidelines and were approved by our Institutional Animal Care Committee. Normal Sprague-Dawley male rats (309 g on average) were anesthetized by inhalation with isoflurane (VEDCO, Inc., St. Joseph, MO) (3% in 100% oxygen). The ^{99m}Tc-Doxil® (2.0 ml) containing 0.45 mCi (16.65 MBq) of ^{99m}Tc, 2 mg of doxorubicin (6.47 mg doxorubicin / kg rat) and 15.96 mg of total lipids or 2.0 ml of free ^{99m}Tc-BMEDA containing 0.45 mCi (16.65 MBq) of ^{99m}Tc was administered to each rat by bolus injection through tail vein. Blood samples (50 µl per sample) were collected from the

tail vein opposite to the administration vein at baseline, 30 min, 2 h, 4 h, 20 h and 44 h after administration. The gamma camera images were acquired at the times immediately after blood collection (Image resolution: 128 × 128. Acquisition time: 1 min each from baseline to 4 h after administration; 5 min at 20 h; 20 min at 44 h).

After blood sample collection and image acquisition at 44 h, anesthetized rats were euthanized by cervical dislocation and the major organs and tissues were collected. Femur with bone marrow was taken as representative of bone and bone marrow. Accumulated bowel or feces activity in 44 h was determined by counting an aliquot of samples after digestion in saturated sodium hydroxide (NaOH). The ^{99m}Tc activity distribution of ^{99m}Tc-Doxil® or free ^{99m}Tc-BMEDA in various rat tissues and the blood samples collected from the tail veins at various times were measured with a Minaxiγ A5550 gamma counter (Packard, Downers Grove, IL). Total blood, bone, muscle and skin mass of rats were calculated as 5.4 %, 10 %, 40 % and 13 % of total body weight, respectively (Frank, 1976). No blood correction was performed with organ activity. The blood clearance pattern of ^{99m}Tc-Doxil® or free ^{99m}Tc-BMEDA was simulated with the following dual-exponential equation:

$$Y = b_1 \times e^{-c_1 \times t} + b_2 \times e^{-c_2 \times t}$$

Here Y is the percent blood ^{99m}Tc activity; t is the time after injection; b_1, b_2, c_1 and C_2 are constants. The two-phase blood clearance half-times ($(t_{1/2})_1$ and $(t_{1/2})_2$) were calculated from the simulated dual-exponential curves as follows:

$$(t_{1/2})_1 = 0.693/c_1;\ (t_{1/2})_2 = 0.693/c_2.$$

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Statistical Analysis. MiniTab program (MiniTab Inc, State College, PA) was used to perform statistical analysis. Origin software (OriginLab Corporation, Northampton, MA) was used to simulate blood clearance patterns and spin column data. All average values are given as mean ± standard deviation (sd). The comparison of % injected dose / organ between ^{99m}Tc-Doxil® and free ^{99m}Tc-BMEDA groups was determined using one-way ANOVA. The acceptable probability for a significant difference was $P < 0.05$.

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Results

Labeling Efficiency of ^{99m}Tc-Doxil®. The labeling efficiency of ^{99m}Tc-Doxil® for *in vitro* stability studies was 70.6 ± 0.8 % (n = 3). The labeling efficiency of ^{99m}Tc-Doxil® for rat distribution study was 39.5 %. After ^{99m}Tc labeling, the average particle size of Doxil® was 89.9 ± 16.2 nm which is similar to the particle sizes of Doxil® before ^{99m}Tc labeling.

***In Vitro* Labeling Stability of ^{99m}Tc-Doxil®.** The spin column elution profiles of ^{99m}Tc activity, doxorubicin and serum protein are shown in Figure 2. Doxorubicin was in red color. After 6th fraction of spin column elution, no doxorubicin was observed. Spectrophotometric measurements of fractions after 6th fraction of elution were at background levels. Assuming ^{99m}Tc activity was associated with the liposomes trapping doxorubicin or with the serum protein at different times of incubation, the simulated curve of ^{99m}Tc activity of the sample after incubation for 72 h is shown in Figure 2. The simulated curve showed very good correlation with ^{99m}Tc activity profile. This suggests that ^{99m}Tc release from liposomes was mediated by serum protein.

The percent of ^{99m}Tc associated with liposomes after incubation in 50 % FBS-PBS buffer, pH 7.4, at 37^{o}C at different times is shown in Figure 3. After 24 h incubation, there was 72.3 ± 3.6 % of ^{99m}Tc activity associated with liposomes. Incubation of ^{99m}Tc-Doxil® in 50 % human serum-PBS buffer, pH 7.4, at 37^{o}C showed that 78.6 ± 1.8 % of ^{99m}Tc activity associated with Doxil® at 24 h and 77.9 ± 0.5 % associated

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with Doxil® at 72 h (n = 3). After storing ^{99m}Tc-Doxil® at 25°C in PBS buffer, pH 7.4, for 24 h, there was 89.4 ± 0.55 % of ^{99m}Tc activity associated with liposomes.

Normal Rat Distribution of ^{99m}Tc-Doxil®. The gamma camera images of ^{99m}Tc-Doxil® labeled with ^{99m}Tc-BMEDA and ^{99m}Tc-BMEDA alone after intravenous bolus injection at various times are shown in Figure 4. ^{99m}Tc-Doxil® showed slow blood clearance and no significant excretion from bowel and bladder. ^{99m}Tc-Doxil® also had a uniform high level of ^{99m}Tc-activity in the intestine and surrounding abdominal tissues at 4 h, 20 h and 44 h. In contrast, for ^{99m}Tc-BMEDA alone, ^{99m}Tc-activity was removed quickly from blood and excreted via bladder and digestive tract.

Figure 5 shows the blood clearance curves of ^{99m}Tc-Doxil® labeled with ^{99m}Tc-BMEDA and ^{99m}Tc-BMEDA alone from baseline to 44 h. Exponential curve-fitting analysis of ^{99m}Tc-Doxil® blood clearance showed a two-phase blood clearance with 36.4 % of the injected activity having a half clearance time of 2.2 h and 63.7 % of injected activity had half clearance time of 26.2 h (n = 5), which is similar to the reported blood clearance characteristics of Doxil® (Gabizon et al., 2003). In contrast, the unencapsulated ^{99m}Tc-BMEDA had very rapid blood clearance with 95.5 % of the injected activity having a half clearance time of only 0.12 h (n = 4).

Figure 6 shows the normal rat distribution of ^{99m}Tc-Doxil® labeled with ^{99m}Tc-BMEDA and ^{99m}Tc-BMEDA alone at 44 h after injection. At 44 h after injection, the ^{99m}Tc activity mainly existed in blood, liver, bone with bone marrow, skin, bowel, and kidney with % injected dose of 19.8 ± 1.3 %, 14.1 ± 1.7 %, 9.0 ± 0.8 %, 6.0 ± 0.5 %, 15.3 ± 4.3 %, and 7.8 ± 0.9 %, respectively. Spleen, blood, liver, bone with bone

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marrow, skin, muscles and bowel showed significantly higher % ^{99m}Tc activity / organ after injection of ^{99m}Tc-Doxil® compared with ^{99m}Tc-BMEDA alone ($P < 0.001$). Rats also had significantly higher level of ^{99m}Tc-Doxil® in the bowel at 44 h after administration ($P < 0.01$). ^{99m}Tc-BMEDA alone had significantly higher excretion from urine and feces ($P < 0.01$).

Discussion

In animal studies, ^{14}C labeled lipids or ^{3}H labeled cholesterol have been used to study the *in vivo* behavior of liposomes (Allen and Hansen, 1991; Unezaki et al., 1994). The advantage of ^{14}C or ^{3}H labeling is that this labeling will not change the chemical structure of lipids, cholesterol or drug molecules, because carbon and hydrogen atoms exist in the original molecules, and the isotope substitution will not change the *in vivo* behaviors of the labeled molecules. The major shortcoming of using ^{14}C or ^{3}H for *in vivo* behavior studies is that they are pure beta emitters and thus cannot be used to non-invasively detect the distribution in different tissues or organs in live animals using imaging. They also have long half lives (^{14}C, 5730 years; ^{3}H, 12.33 years), so that it is not feasible to use them in human subjects.

To achieve liposome labeling, radioisotopes can be attached to the surface of a liposome, intercalated into the double membrane of a liposome or encapsulated within the inner space of a liposome (Hafeli et al., 1991). To study the pharmacokinetics of liposomal therapeutic drugs indirectly by tracking the *in vivo* behavior of liposomes, an ideal labeling method is the direct radiolabeling of a liposome that is already loaded with drug molecules. This method would be the least likely to influence the *in vivo* behavior of liposomes that encapsulate therapeutic drugs. The direct radiolabeling of liposomes using an existing pH or ammonium gradient in liposomes carrying therapeutic drugs is convenient for the study of commercially available liposome formulations. To achieve this goal, a radiolabeled compound with certain lipophilicity is required for the compound to move across the lipophilic double membrane. Once in the liposome interior, the radiolabeled compound can be

protonized in the lower pH environment of the inner space and be trapped. The ^{99m}Tc-BMEDA complex we have studied is neutral and has certain lipophilicity at a higher pH environment. ^{99m}Tc-BMEDA also contains amine groups, so that it can be trapped within a liposome using a pH or ammonium gradient mechanism. These mechanisms enable the direct radiolabeling of the commercial Doxil® with ^{99m}Tc-BMEDA.

Until now, no satisfactory method of directly labeling pH gradient or ammonium gradient liposomes encapsulating therapeutic drugs with ^{99m}Tc has been reported. Another group has reported the direct labeling of liposomes using ^{99m}Tc-DTPA, a very hydrophilic complex. The effectiveness of this labeling method and the correlated imaging studies are debatable (Laverman et al., 2002). A reported Doxil® direct labeling method using ^{111}In-oxine to label Doxil® with ^{111}In was reported by Laverman et al (Laverman et al., 2001; Laverman et al., 2002). By using this labeling method, gamma camera images up to 4 h after administration were reported, however no information describing the labeling stability of this labeling method was reported.

Animal and human pharmacokinetic studies on Doxil® showed that over 95 % of doxorubicin stays trapped in liposomes in plasma (Gabizon et al., 2003). This suggests the importance of clarifying the *in vivo* behavior of pegylated liposomes encapsulating therapeutic drugs. These pharmacokinetic studies demonstrated that the injected lipid amount will not influence the pharmacokinetics of pegylated liposomes if the injected lipids in the liposomes are less than 400 μmole / kg (Allen and Hansen, 1991). The injected total lipid dose in our study was 46.3 μmole / kg. Previous animal and human subject studies have shown that Doxil® has a two-phase blood clearance with an initial phase half clearance time of 1-3 h and a second phase half clearance

time of 30-90 h (Gabizon et al., 2003). Our normal rat study showed a two-phase blood clearance pattern with first phase half clearance time of 2.2 h and second phase half clearance time of 26.2 h (Fig. 5). These normal rat distribution results of ^{99m}Tc-Doxil® labeled with ^{99m}Tc-BMEDA are compatible with the data reported previously. In contrast, free ^{99m}Tc-BMEDA has a very short blood half-clearance time of only 0.12 h.

The gamma camera images of the ^{99m}Tc-Doxil® at different times in the current study depict the slow blood clearance and low level of excretion from bladder and digestive tract (Fig. 4). In addition, ^{99m}Tc-Doxil® also accumulated in the intestine and the surrounding tissues which is visible beginning 4 h after administration. The intestinal distribution of ^{99m}Tc-Doxil® is very different from the free ^{99m}Tc-BMEDA which had fast excretion from the bladder and digestive tract but did not distribute uniformly in the intestine and surrounding tissues. Normal rat distribution at 44 h showed that ^{99m}Tc-Doxil® has high levels of activity in blood, liver, bone with bone marrow, skin, and bowel. These results are also similar to the biodistribution of Doxil® reported previously (Gabizon et al., 2003). Comparison between ^{99m}Tc-Doxil® labeled with ^{99m}Tc-BMEDA and free ^{99m}Tc-BMEDA showed that normal rats injected with ^{99m}Tc-Doxil® had significantly higher activity in blood, spleen, liver, bone with bone marrow, skin, muscle ($P < 0.001$ for all above organs), and bowel ($P < 0.01$). The relative high concentration of Doxil® in skin and bowel may be related to the cutaneous and digestive system toxicities, such as mucositis and diarrhea, which has been reported in clinical trials (Tsavaris et al., 2002; Syrigos et al., 2002; Androulakis et al., 2002; Skubitz, 2003). Because Doxil® is a pegylated liposome, we observed

less activity in the spleen compared with liposomes without polyethylene glycol (PEG) (Bao et al., 2003a).

The biodistribution showed no significant difference in kidney activity between ^{99m}Tc-Doxil® labeled with ^{99m}Tc-BMEDA and free ^{99m}Tc-BMEDA ($P = 0.075$). This suggests that after 44 h, some of the ^{99m}Tc-BMEDA released from metabolized liposomes accumulated in the kidney. Prior studies have established that liposomes do not distribute to any extent within the kidneys. This suggests that there is a need to be cautious when interpreting the biodistribution of the pegylated liposomes in kidney using the ^{99m}Tc-liposomes labeled with ^{99m}Tc-BMEDA, particularly in studies of greater than 4 hours after administration when post-metabolism of ^{99m}Tc-BMEDA begins to occur.

Tumor-bearing animal and cancer patient studies showed that pegylated liposomes had significant localization in the tumors (Gabizon, 1992; Harrington, et al., 2001). The labeling of liposomes with ^{99m}Tc-BMEDA has the potential for studying the tumor localization of a variety of pegylated liposomes encapsulating therapeutic drugs.

Imaging of the actual distribution of liposomes encapsulating drug molecules post-therapy could verify tumor drug delivery. A low liposome accumulation in a particular patient's tumor may explain a lack of therapeutic response. The studies on the relationship between intratumoral Doxil® concentration and treatment response would be beneficial to understand the pharmacokinetics of Doxil® and to determine the proper Doxil® dose to treat a tumor with good treatment response while lowering complications. In addition, the use of this imaging technique could also make it

possible to observe possible increased immune responses by the liver and spleen, such as the increased Kupffer cell phagocytic activity (Daemen et al., 1995).

This non-invasive imaging method may also be useful for studying the distribution of Doxil® after an intervention aimed at increasing liposome accumulation in tumors. These interventions include focused hyperthermia, radio-frequency ablation and radiation (Koukourakis et al., 2000; Matteucci et al., 2000; Monsky et al., 2002). These physical modalities have been shown to increase liposome tumor accumulation by 2 to 10 fold following intravenous administration. This labeling method also makes it possible to study the tumor retention and distribution after direct intratumoral injection of ^{99m}Tc-Doxil® (Harrington et al., 2000c).

Another potential use of this methodology could be to label Doxil® with therapeutic radionuclides for combined chemotherapy and radionuclide therapy. There are two therapeutic radionuclides, ^{186}Re and ^{188}Re, which belong to the same elemental group as technetium. Studies have shown that ^{99m}Tc-"SNS/S" and Re-"SNS/S" complexes have the same coordinate structures (Pirmettis et al., 1996; Pelecanou et al., 1999). In previous research, we have also demonstrated that ammonium gradient liposomes can also be labeled with ^{186}Re and ^{188}Re using ^{186}Re / ^{188}Re-BMEDA (Bao et al., 2003b).

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Figure Legends

Figure 1. Chemical structure of ^{99m}Tc-BMEDA.

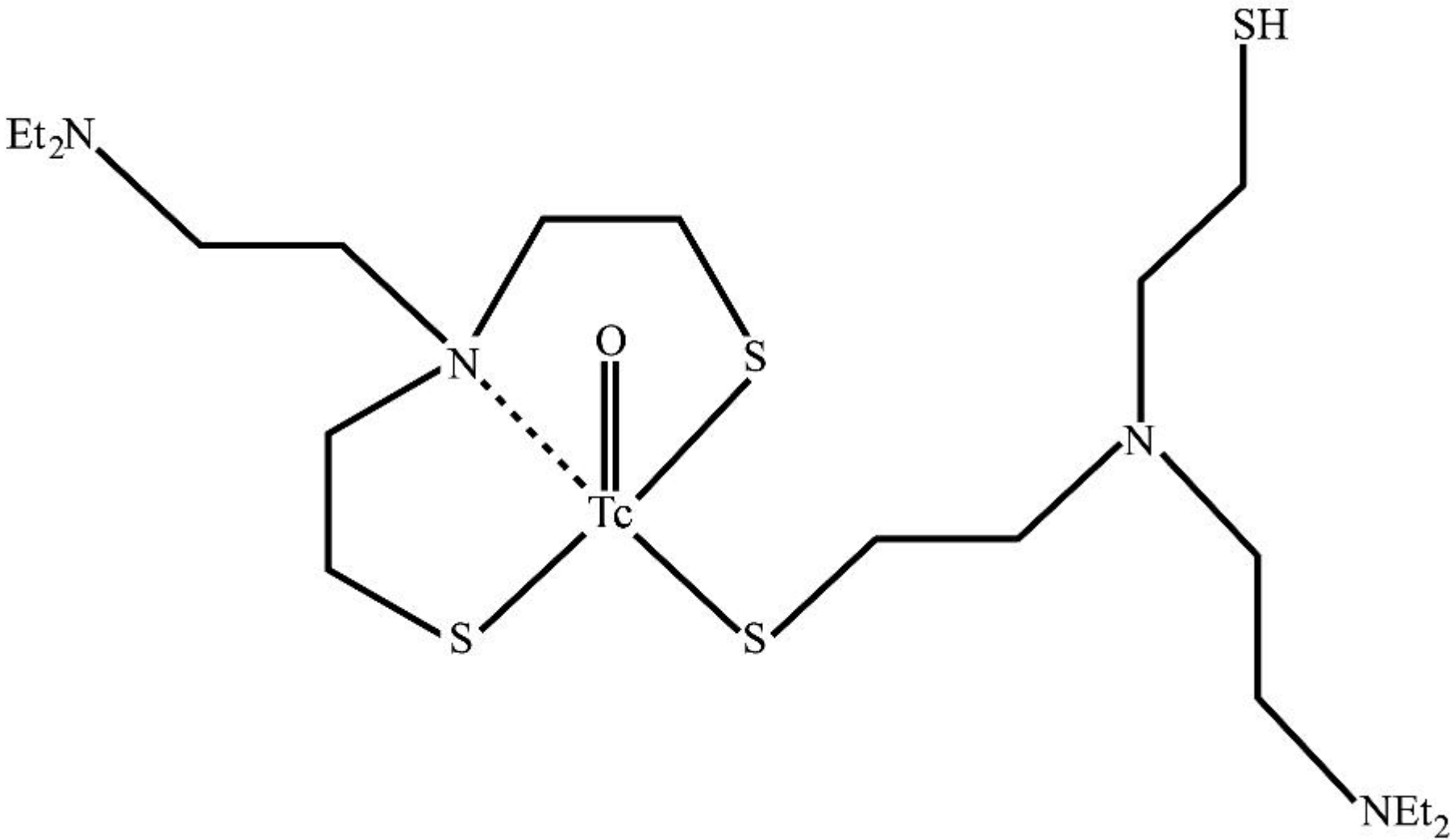
Figure 2. The elution profiles of ^{99m}Tc activity, doxorubicin and serum protein. The sample depicted is from a ^{99m}Tc-Doxil® serum aliquot after incubation at 37^{o}C for 72 h. Because of the large particle size of the pegylated liposomes trapping doxorubicin, Doxil® was eluted first from the spin column. No significant doxorubicin was left in the column after 20 fractions. From the doxorubicin profile, most of doxorubicin was associated with liposomes. The simulated curve is the simulated ^{99m}Tc activity profile with the assumption that ^{99m}Tc activity was associated with pegylated liposomes or associated with serum protein. The simulated curve fits with ^{99m}Tc activity curve very well (Paired t test, P = 0.89).

Figure 3. Per cent ^{99m}Tc activity associated with liposomes at different times after incubation with 50 % FBS-PBS buffer, pH 7.4, at 37^{o}C (n = 3).

Figure 4. Gamma camera images depicting normal rat distribution of ^{99m}Tc-Doxil® labeled with ^{99m}Tc-BMEDA and ^{99m}Tc-BMEDA alone at various times after injection.

Figure 5. Blood clearance curves of ^{99m}Tc-Doxil® labeled with ^{99m}Tc-BMEDA and ^{99m}Tc-BMEDA alone. The fitted curves are dual-exponential decay curves.

Figure 6. Normal rat distribution of ^{99m}Tc-Doxil® labeled with ^{99m}Tc-BMEDA and ^{99m}Tc-BMEDA alone at 44 h after intravenous bolus injection. The feces and urine activities were accumulated activity in 44 hours. One-Way ANOVA is used to compare ^{99m}Tc-Doxil® and ^{99m}Tc-BMEDA alone, ***, P < 0.001; **, P < 0.01.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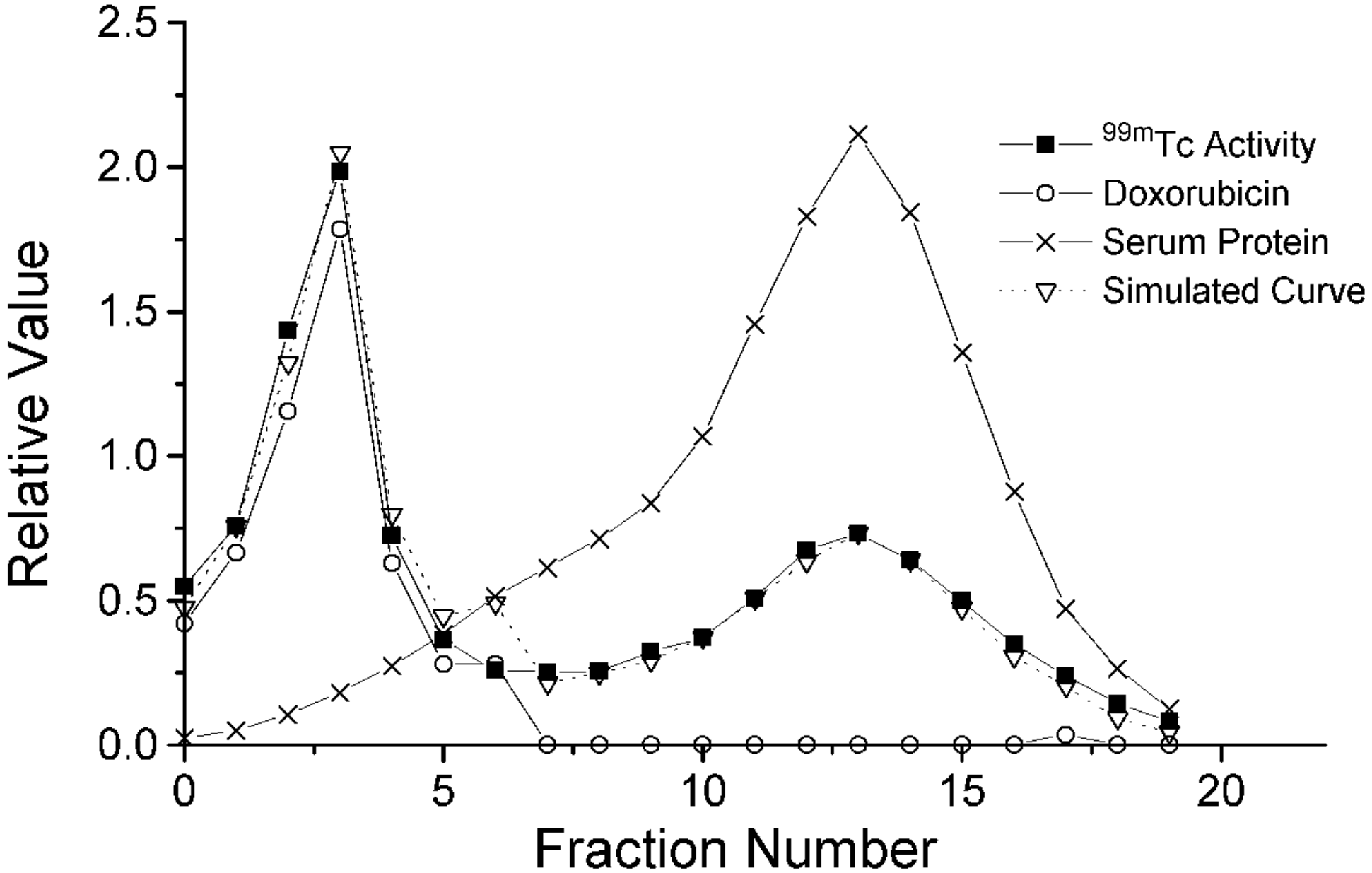
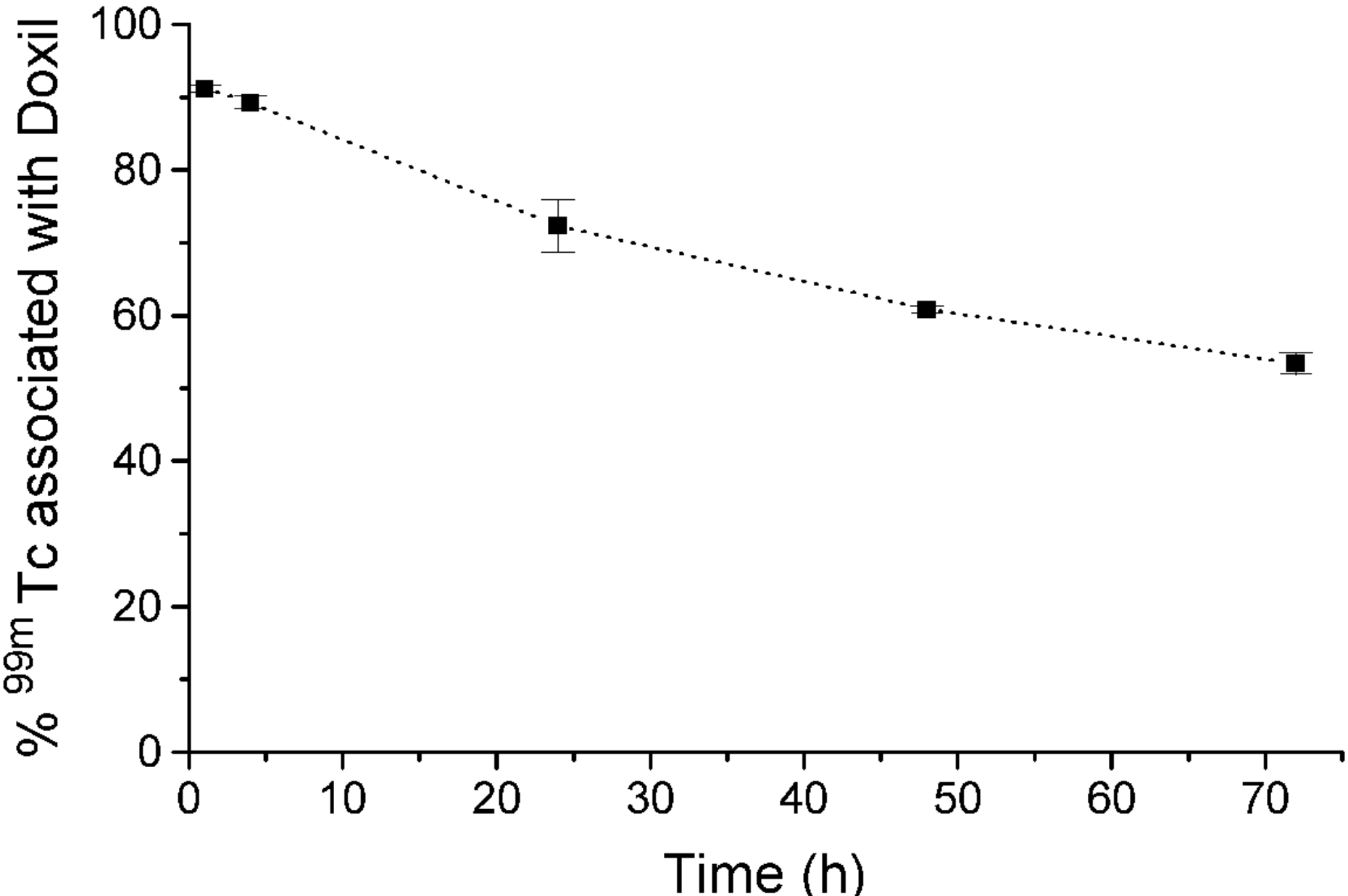
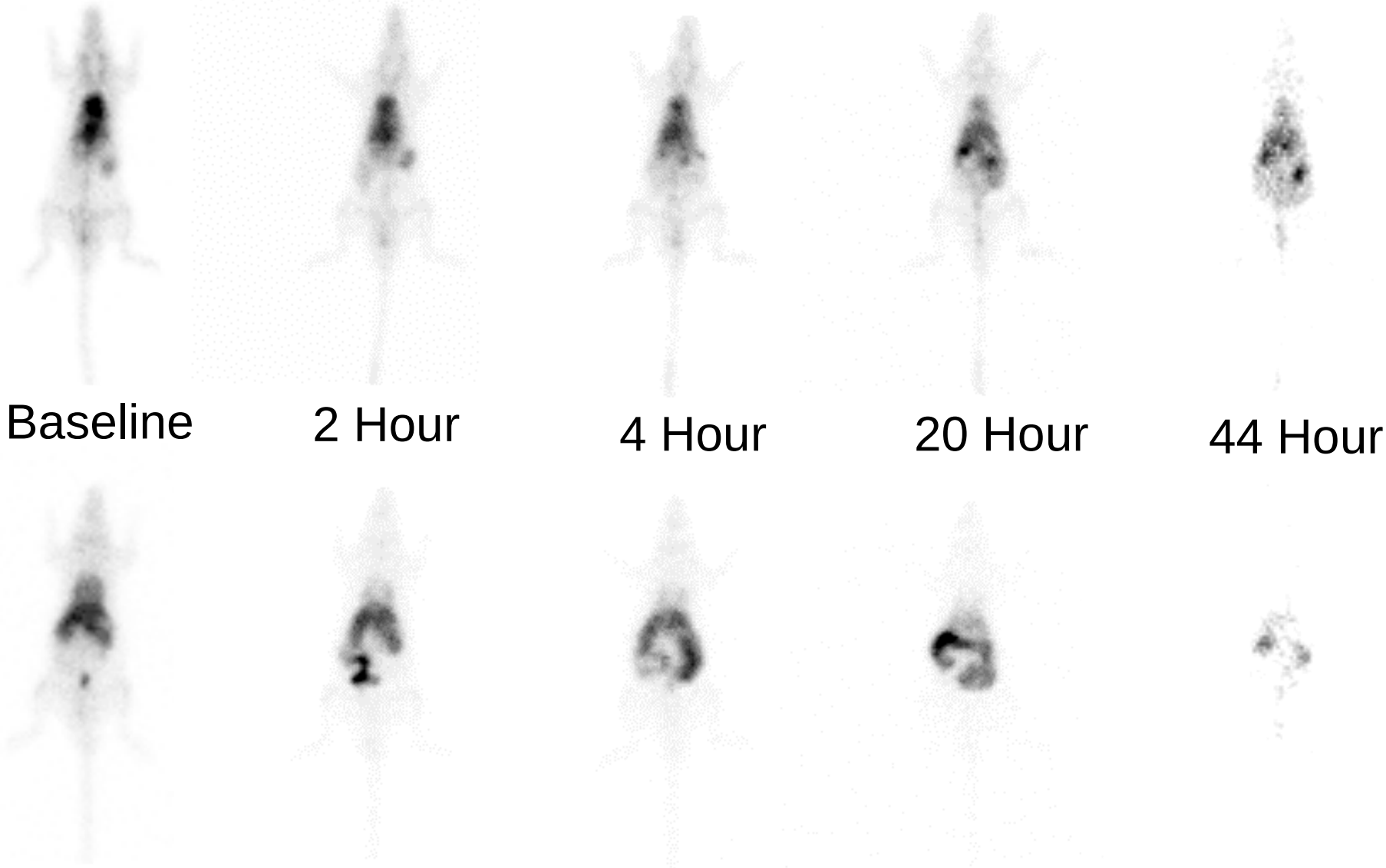
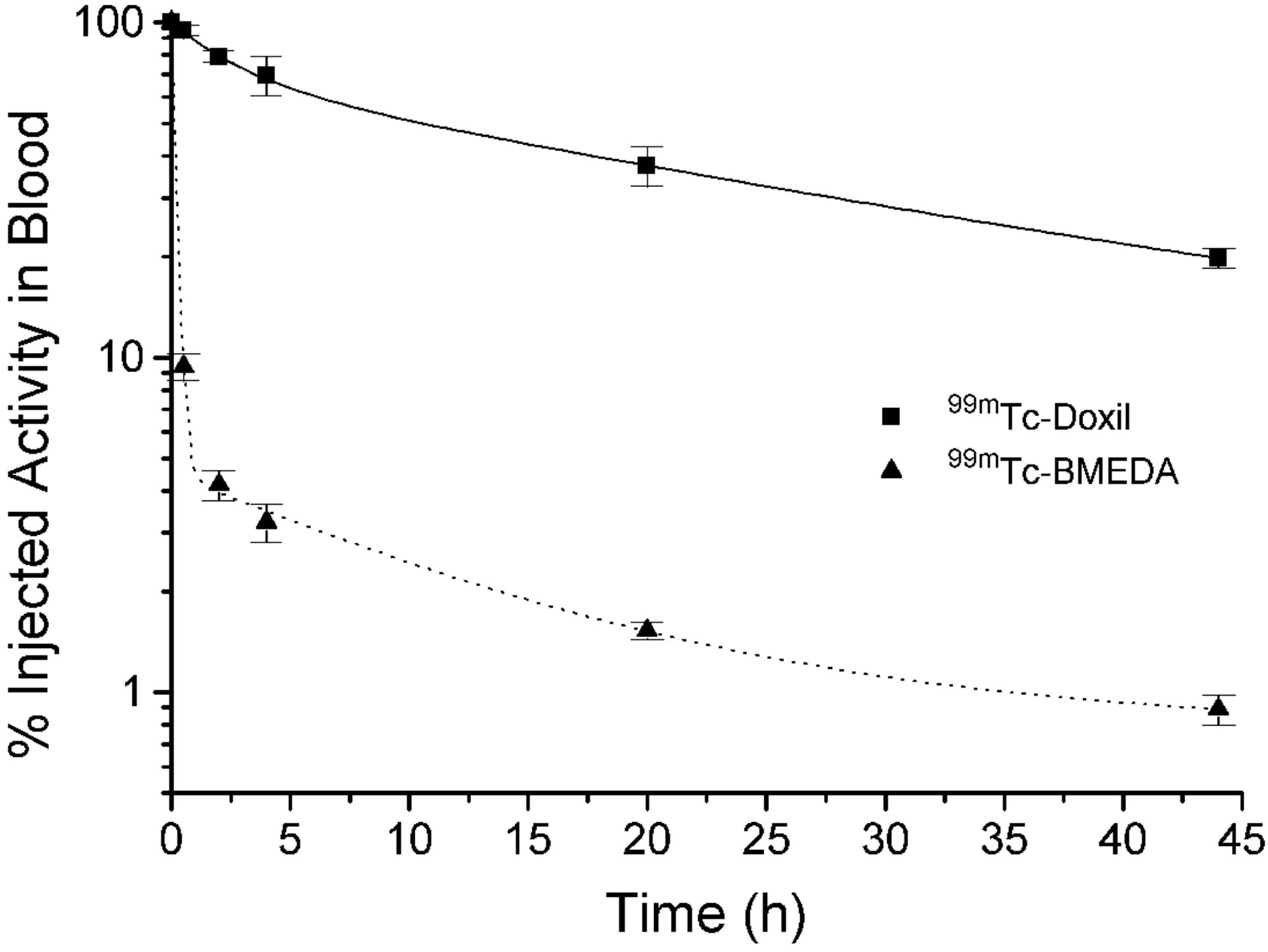

Relative Value
Fraction Number
0.0
0.5
1.0
1.5
2.0
2.5
0
5
10
15
20
99mTc Activity
Doxorubicin
Serum Protein
Simulated Curve

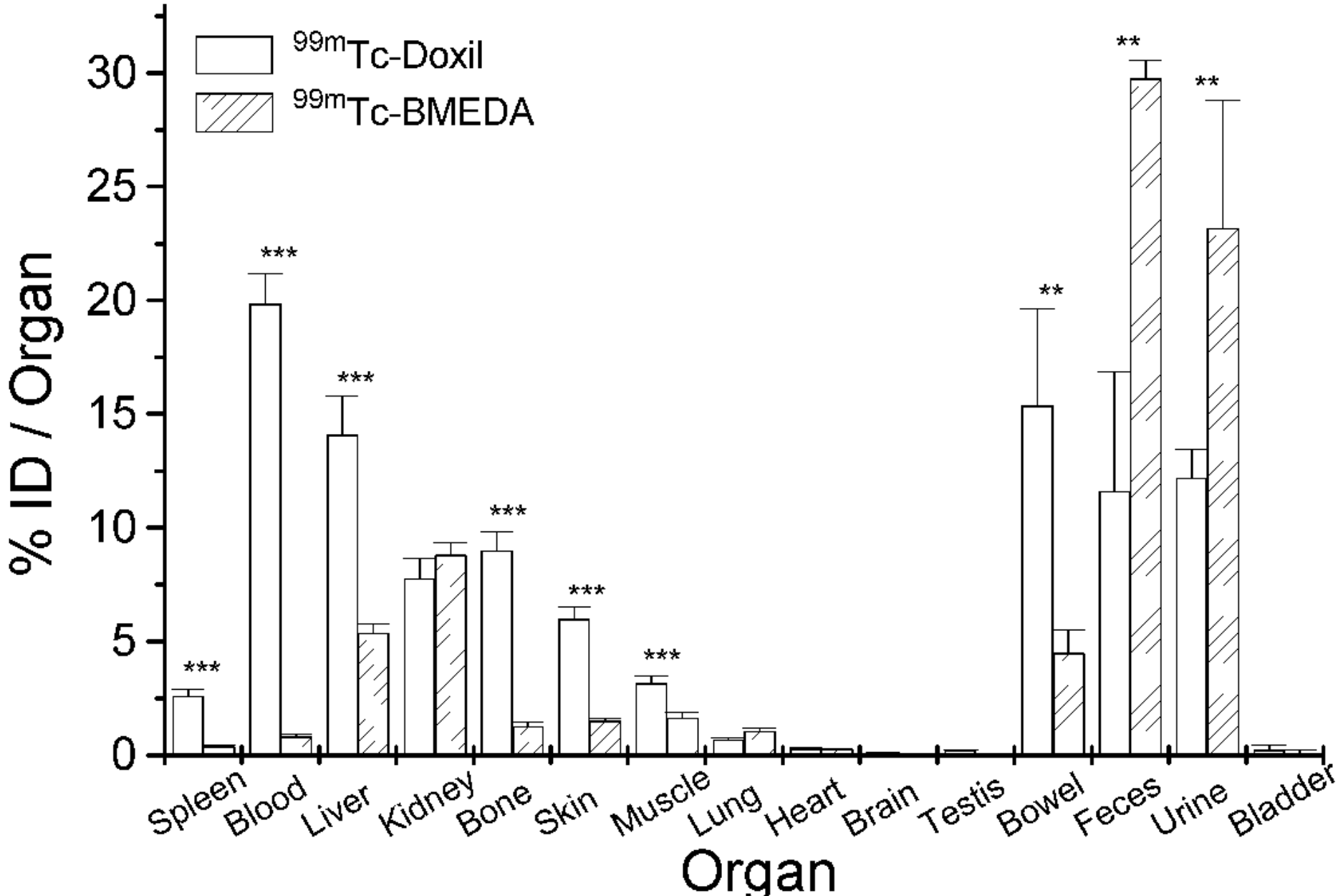

% 99m Tc associated with Doxil
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Time (h)

^{99m}Tc-Doxil® Labeled with ^{99m}Tc-BMEDA



^{99m}Tc-BMEDA Alone


100
10
1
% Injected Activity in Blood
0
5
10
15
20
25
30
35
40
45
Time (h)
99mTc-Doxil
99mTc-BMEDA


99mTc-Doxil
99mTc-BMEDA
% ID / Organ
0
5
10
15
20
25
30
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**
Spleen
Blood
Liver
Kidney
Bone
Skin
Muscle
Lung
Heart
Brain
Testis
Bowel
Feces
Urine
Bladder
Organ