Evaluation of Tc-99m (V) DMSA Binding to Human Plasma Proteins

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As a critical step toward elucidating the mechanism of localization of Tc-99m (V) dimercaptosuccinic acid (DMSA), we investigated its binding and transport in blood in comparison with Ga-67 citrate. The studies were performed *in vitro* by incubating Tc-99m (V) DMSA with blood (one sample at 4°C and another at 37°C) to assess its binding to plasma proteins using ultrafiltration, dialysis, electrophoresis, gel filtration chromatography and affinity chromatography. A parallel experiment for determining the blood binding of Ga-67 citrate was performed using the same procedures. Using ultrafiltration, dialysis, electrophoresis and gel filtration chromatography, labeled plasma samples showed that protein binding for Tc-99m (V) DMSA was 45–54% at 37°C and 73–80% at 4°C. The figures for Ga-67 citrate were 43–53% at 37°C and 75–81% at 4°C. Electrophoresis showed that Tc-99m (V) DMSA was mostly bound to plasma albumin (36.05 ± 2.48% at 37°C and 60.04 ± 1.87% at 4°C), and that the proportion of Ga-67 radioactivity associated with β-globulin was 34.23 ± 1.37% at 37°C and 55.71 ± 3.69% at 4°C. In affinity chromatography experiments, Tc-99m (V) DMSA did not bind to transferrin, unlike Ga-67 citrate. This study demonstrates that, at the radiopharmaceutical tracer level, most Tc-99m (V) DMSA in blood is protein-bound, primarily to albumin, but not to transferrin. In contrast, Ga-67 citrate was bound primarily to transferrin. The knowledge that albumin is the main transport protein of Tc-99m (V) DMSA may contribute to a better understanding of its biodistribution and pharmacokinetics.

Key Words: albumin, Ga-67 citrate, plasma proteins, Tc-99m (V) DMSA, transferrin
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The pentavalent Tc-99m dimercaptosuccinic acid (Tc-99m (V) DMSA, Tc(V)-DMSA) radiotracer has become more widely used. The accumulation of Tc(V)-DMSA in tumors was first reported in 1981 [1], and this tracer has subsequently been shown to localize in a variety of tumors [2,3]. Tc(V)-DMSA has also been used to depict abscesses and other inflammatory lesions [4–6]. Compared with conventional inflammation and the tumor-imaging agent Ga-67 citrate (Ga-citrate), Tc(V)-DMSA has superior characteristics, such as better physical properties, lower cost, and less time delay between radiotracer injection and imaging. In addition, compared with some recently developed radiopharmaceuticals for oncologic imaging, such as monoclonal antibodies, metabolic substrates and receptor-avid peptides, Tc(V)-DMSA remains cost-effective, widely available and clinically accepted.

Despite years of experience with Tc(V)-DMSA imaging, the precise mechanism by which Tc(V)-DMSA

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accumulates remains controversial. The chemical identity of Tc(V)-DMSA was determined using analytical methods [7]. It has been suggested the tumor affinity of Tc(V)-DMSA can be attributed to the structural similarity of the TcO_4^{-3} core to the PO_4^{-3} ion [8]. A pH-dependent mechanism has also been advanced, based on evidence of increased accumulation of Tc(V)-DMSA in tumor cells with acidification both *in vitro* and *in vivo* [9,10]. In addition, Tc(V)-DMSA has an affinity for calcified materials [11]. Watkinson et al [12] showed that Tc(V)-DMSA has a bioexponential blood clearance, but did not mention blood binding or transport. Furthermore, Tc(V)-DMSA enters cancer-cell-line models, specifically via the type III NaPi co-transporter (PiTl) [13]. PiTl activity is inhibited during the early stages of apoptosis, leading to differential incorporation of Tc(V)-DMSA in viable cells and apoptotic cells, both *in vitro* and *in vivo* [14]. Yet, to the best of our knowledge, there are no published studies on the specific binding of Tc(V)-DMSA to specific classes of proteins.

The interaction of different drugs with circulatory proteins is of great importance because this influences both their distribution in the body and the rate of their elimination. Therefore, we aimed to study the interactions of Tc(V)-DMSA with blood, to ascertain which plasma proteins transported Tc(V)-DMSA, and to determine what influences the biodistribution of Tc(V)-DMSA. We hypothesized this information might lead to improved imaging and therapeutic results, since the pharmacokinetic properties of Tc(V)-DMSA and Re-186 (V) DMSA are similar [15].

There are similarities and differences between the clinical imaging diagnosis of Tc(V)-DMSA and Ga-citrate. Both Tc(V)-DMSA and Ga-citrate images showed abnormal uptake in some tumors [2,3] and inflammatory lesions [4–6]. However, Tc(V)-DMSA scans are positive and Ga-citrate scans are negative in schwannomas [2] and mediastinal neurilemmomas [3]. Several investigators have reported that serum transferrin transports Ga-citrate to tumor tissues [16–18]. Transferrin receptors on tumor cells may be involved in tumor accumulation [17]. Moreover, hypotheses for the tumor uptake of Ga-67 include endocytosis of protein-bound gallium by tumor cells, diffusion across hyperpermeable tumor cells and plasma membranes, and exchange of transferrin-bound gallium with lactoferrin. We investigated whether Tc(V)-DMSA and Ga-citrate manifested similar or different biological properties in terms of blood binding and transport. The specific nature of Tc(V)-DMSA binding to particular plasma proteins has not yet been established.

To understand the mechanism of localization of Tc(V)-DMSA in specific target tissues, the interactions of Tc(V)-DMSA with plasma proteins are important. We examined two aspects of the interactions of Tc(V)-DMSA: total protein binding and the specificity of binding to certain classes of proteins. Using the same procedures, we also studied the binding of Ga-citrate to plasma proteins for comparison and to validate the experimental procedures by checking their agreement with previous reports [16–18].

Materials and Methods

Preparing radiopharmaceuticals

Tc(V)-DMSA was prepared by adding 370 MBq of sodium pertechnetate Tc-99m into a commercially available Tc(V)-DMSA kit (INER DMS Kit; INER, Taoyuan, Taiwan) containing 1.0 mg of DMSA, 5.0 mg of sodium bicarbonate, 9.0 mg of sodium chloride, 1.0 mg of ascorbic acid and 0.5 mg of stannous chloride per vial. The radiochemical purity of the Tc(V)-DMSA was checked using instant thin-layer chromatography. Labeling purity was >95% for each preparation. The radiotracer Ga-citrate was obtained from the Radiochemical Center (DuPont, Billerica, MA, USA).

Radioactivity in blood cells and plasma

Between August 2000 and July 2002, 36 healthy volunteers (18 men, 18 women; weight range, 60–85 kg; age range, 45–60 years) were fasted for about 11 hours. Eight-milliliter blood samples were drawn from the left forearm vein into lithium-heparinized vacutainers. We took triplicate 2-mL samples from each whole-blood sample and added 370 kBq of Tc(V)-DMSA. After 2 hours of incubation (one sample at 4°C and another at 37°C), the labeled samples were centrifuged at 3,500 rpm for 10 minutes at room temperature (approximately 25°C). The radioactivities of packed blood cells and supernatant were measured with a gamma counter (COBRA II; Packard, Meriden, CT, USA). The relative distribution of radioactivity between the packed cells and the plasma was determined. As a comparison, Ga-citrate was identically analyzed.

The remaining labeled plasma from each whole-blood sample was tested to determine total protein

binding as well as the relative distribution of Tc(V)-DMSA among the major protein fractions. Due to logistical limitations, not all tests could be performed on all labeled plasma samples. The same procedures were performed for Ga-67. All tests were performed twice to confirm the reproducibility of the results.

Total protein binding: ultrafiltration

A 0.2-mL sample of labeled plasma was placed in a membrane ultrafiltration cone (Amicon Corp., Lexington, MA, USA) for retention of molecules above 25,000 Da. The cones were centrifuged at 3,000 rpm for 15 minutes and centrifuged again after adding 0.2 mL of water (pH 7.5), following which the radioactivities of filtrates and residues were measured using a gamma counter. Total protein binding was calculated as a percentage of the radioactivity in the applied plasma sample retained in the cone. As a control, a similar procedure was followed for Tc(V)-DMSA alone, and the percentage of radioactivity bound to the cone was determined.

Total protein binding: dialysis

A 1-mL labeled plasma sample was dialyzed at 4°C for 24 hours in physiological saline (pH 7.4), using a dialysis membrane that retained molecules above 12,000 Da. After dialysis, the contents of the dialysis bag and the dialyzed fluid were taken and counted using a gamma counter. Total protein binding was calculated as a percentage of the applied plasma radioactivity retained inside the membrane. As a control, Tc(V)-DMSA alone samples were dialyzed in the same manner.

Total protein binding: gel filtration chromatography

A 1.5 × 30-cm gel column (Sephadex G-50; Pharmacia Fine Chemicals, Inc., Piscataway, NJ, USA) was prepared and equilibrated with a 0.05-M sodium bicarbonate buffer (pH 7.5). Transferrin (1 mg) was passed through the column as a routine precaution to block any protein binding sites on the gel. A 0.1-mL sample of labeled plasma was applied to the column and eluted with the bicarbonate buffer. Column eluates were consecutively collected and radioactivities measured using a gamma counter. As a control, the elution patterns of Tc(V)-DMSA alone were determined. Total protein binding was calculated as a percentage of the applied radioactivity that appeared in the protein region in the void volume. The total radioactivity recovered from the column, expressed as a percentage of the applied radioactivity, was also determined.

Distribution among plasma proteins: agarose gel electrophoresis

We used agarose gel electrophoresis to separate and quantify plasma proteins. Labeled plasma samples (3 μL) were placed onto an agarose plate and allowed to diffuse into the agarose for 4 minutes. Electrophoresis was run at 120 volts for 15 minutes in TITAN GEL Plasma Protein Buffer (Helena Laboratories, Beaumont, TX, USA). As a control, the electrophoretic behavior of Tc(V)-DMSA alone was determined. At the end of each run, the electrophoretograms were stained with amido black and destained in 7% acetic acid. The five protein bands (albumin, and alpha$_1$ (α_1), alpha$_2$ (α_2), beta (β) and gamma (γ) globulins) observed following gel electrophoresis were individually cut out and their radioactivities measured, along with any radioactivity that had migrated beyond the albumin band, using a gamma counter. The relative distribution of Tc(V)-DMSA in the applied plasma sample was calculated as a percentage of the radioactivity that appeared in each electrophoretic fraction. The total radioactivity recovered on the agarose plate, expressed as a percentage of the applied radioactivity, was also determined.

Specific transferrin binding: affinity chromatography

The IgG fraction of sheep anti-human transferrin (4.3 mg) (Serotec, Raleigh, NC, USA) was incubated with cyanogen-bromide-activated Sepharose 4B (1 g) (Pharmacia) for 2 hours in a coupling buffer (0.1 M sodium bicarbonate plus 0.5 M sodium chloride; pH 8.3). Unbound antibody was removed by washing the gel with the coupling buffer, after which the unoccupied binding sites on the Sepharose gel were inactivated by incubating it overnight with 1-M glycine (pH 8.5). The gel was then washed with the coupling buffer (pH 8.3) and, finally, with alternate portions of 0.1-M acetate buffer (pH 4.0) and 0.1-M borate buffer (pH 8.0). Columns with 2-mL bed volumes were prepared from disposable 3-mL plastic syringes. A 0.05-mL labeled plasma sample was applied to the column, incubated with the gel for 15 minutes, and then eluted with normal saline (pH 8.0) in 1-mL increments until no further radioactivity appeared in the

eluate. As a control, Tc(V)-DMSA alone samples were applied to the column, incubated, and eluted in the same fashion. Binding to transferrin was calculated as a percentage of the applied radioactivity retained on the column. The efficiency of transferrin retention by the column was determined to be 98% using I-125 transferrin. To dissociate the antigen–antibody complex and recover the radioactivity retained by the gel, the column was thoroughly washed with 3 M of guanidine hydrochloride (pH 2.0).

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistical comparisons were performed using Student's *t* test with a significance level of 0.05.

Results

Radioactivity in blood cells and plasma

After human whole blood samples with Tc(V)-DMSA or Ga-citrate had been incubated for 2 hours, the relative distribution of radioactivity in blood cells and plasma was similar for both radiotracers, and both showed statistically significant ($p < 0.05$) differences between the levels in blood cells and plasma at 37°C and 4°C (Table 1).

Total protein binding: ultrafiltration and dialysis

Ultrafiltration and dialysis experiments indicated that 60–75% of the Tc(V)-DMSA and Ga-citrate was protein-bound (Table 2). In contrast, little radioactivity was retained in the membrane ultrafiltration cone or the dialysis membrane after Tc(V)-DMSA alone or Ga-citrate alone had been separately analyzed (data not shown).

Total protein binding: gel filtration chromatography

On the elution profile obtained using Sephadex G-50 filtration, Tc(V)-DMSA alone samples showed a single distinct peak, well resolved from the protein peaks in the void volumes at 37°C (Figure A) and 4°C (data not shown). Ga-citrate alone samples at 37°C (Figure B) and 4°C (data not shown) showed similar results. The levels of protein binding were calculated to be 54.12 ± 3.21% at 37°C and 73.24 ± 2.82% at 4°C for Tc(V)-DMSA plasma samples, and 52.96 ± 1.89% at 37°C and 70.18 ± 2.11% at 4°C for Ga-citrate plasma samples.

Distribution among plasma proteins: agarose gel electrophoresis

The characteristic plasma protein fractionation pattern obtained using agarose gel electrophoresis included

Table 1. Relative distribution of radioactivity in blood cells and plasma*

	Blood cells		Plasma	
	37°C	4°C	37°C	4°C
Agent				
Tc(V)-DMSA	22.17 ± 1.47†	15.67 ± 1.63†	78.83 ± 1.72†	82.83 ± 1.47†
Ga-citrate	20.83 ± 0.75†	17.50 ± 1.87†	79.33 ± 2.16†	83.17 ± 2.32†

*Data are expressed as % ± standard deviation, $n = 6$; †$p < 0.05$ between 37°C and 4°C (Student's *t* test).

Table 2. Tc(V)-DMSA and Ga-citrate bound to plasma proteins: based on ultrafiltration and dialysis*

	Ultrafiltration		Dialysis	
	37°C	4°C	37°C	4°C
Agent				
Tc(V)-DMSA	52.83 ± 2.86†	79.83 ± 1.47†	44.50 ± 1.64†	77.67 ± 1.51†
Ga-citrate	52.17 ± 2.64†	60.04 ± 1.87†	43.50 ± 2.43†	74.67 ± 1.63†

*Data are expressed as % ± standard deviation, $n = 6$; †$p < 0.05$ between 37°C and 4°C (Student's *t* test).

five bands representing albumin and the α_1, α_2, β and γ globulins (Table 3). Radioactivity with greater mobility than albumin was considered to be unbound Tc(V)-DMSA. The proportion of bound Tc(V)-DMSA radioactivity associated with the albumin fraction was 36.05±2.48% at 37°C and 60.04±1.87% at 4°C; for the other four fractions, the proportions bound ranged between 2% and 3% (Table 3). The proportion of Ga-citrate radioactivity associated with the β band was 34.23±1.37% at 37°C and 55.71±3.69% at 4°C; for the other four fractions, the proportions bound ranged between 4% and 6%. These results revealed that Tc(V)-DMSA bound primarily to plasma albumin and that Ga-citrate bound primarily to plasma β globulin.

Specific transferrin binding: affinity chromatography

In the affinity chromatography experiment, 87.50± 1.87% at 37°C and 95.50±1.87% at 4°C of the applied Ga-citrate plasma radioactivity was retained by the column and, thus, was bound to transferrin. When

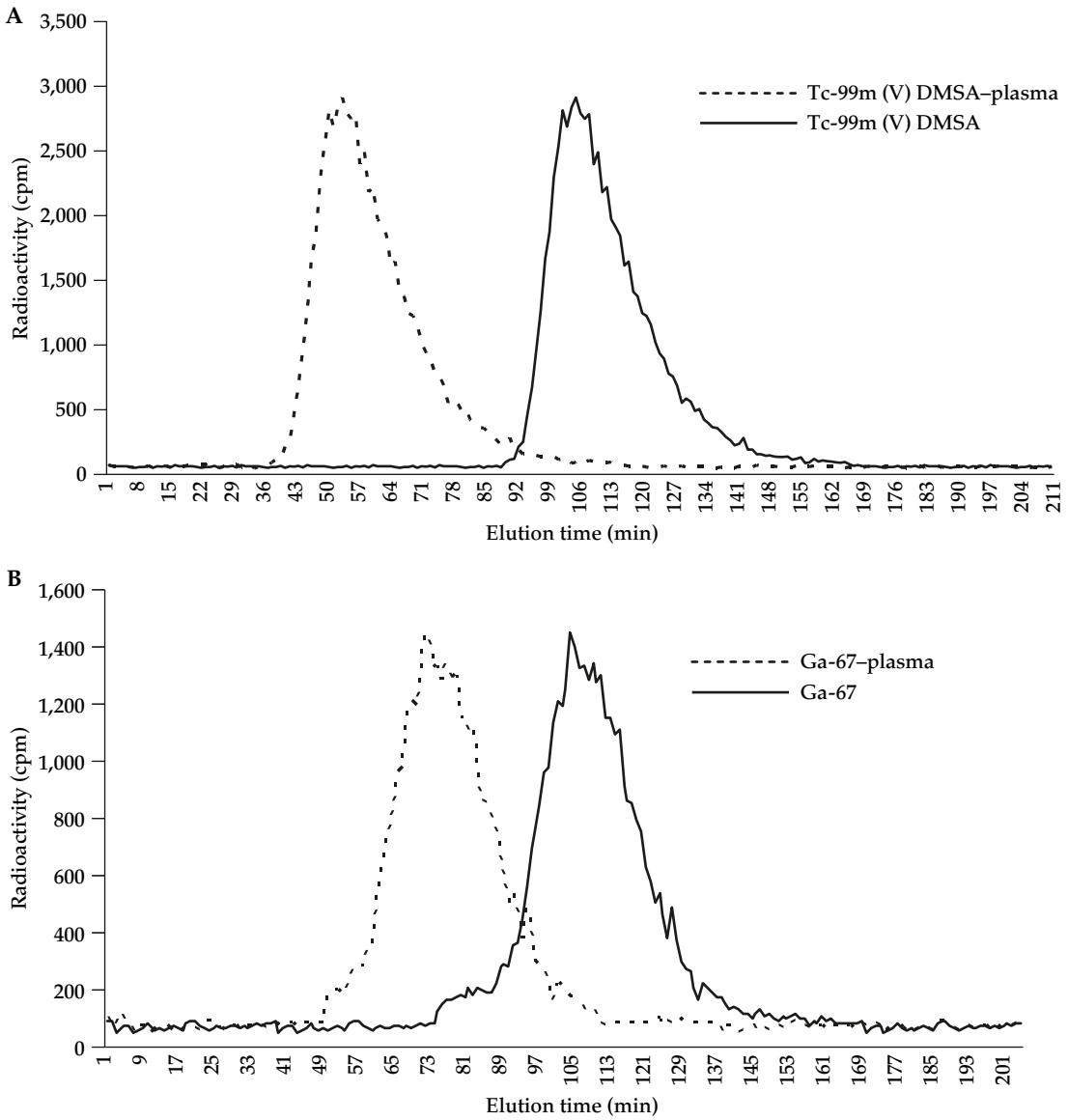

Figure. *(A) A representative example of a Sephadex G-50 gel filtration chromatogram of a Tc-99m (V) DMSA plasma sample (dotted line) and the elution pattern of a Tc-99m (V) DMSA alone sample (solid line), both at 37°C. Protein binding was calculated to be 54.12±3.21% at 37°C and 73.24±2.82% at 4°C. (B) A representative example of a Sephadex G-50 gel filtration chromatogram of a Ga-citrate plasma sample (dotted line) and the elution pattern of a Ga-citrate alone sample (solid line), both at 37°C. Protein binding was calculated to be 52.96±1.89% at 37°C and 70.18±2.11% at 4°C.*

Table 3. Agarose electrophoresis of plasma: Tc-99m (V) DMSA and Ga-citrate distribution in various plasma proteins*

Agent	Incubation temperature	% Total protein binding	% Activity associated with each protein band					% Unbound activity
			γ	β	α_2	α_1	Albumin	
Tc(V)-DMSA	37°C	44.50 ± 2.35†	2.00 ± 1.05	2.15 ± 1.72	2.08 ± 1.60	2.23 ± 0.89	36.05 ± 2.48†	55.50 ± 2.35†
	4°C	76.33 ± 1.63†	3.56 ± 1.03	3.95 ± 1.72	4.45 ± 0.75	4.33 ± 1.03	60.04 ± 1.87†	23.67 ± 1.63†
Ga-citrate	37°C	43.33 ± 3.83†	2.43 ± 1.47	34.23 ± 1.37†	2.36 ± 1.87	2.14 ± 2.37	2.17 ± 1.09	56.67 ± 3.83†
	4°C	75.28 ± 3.50†	5.01 ± 1.47	55.71 ± 3.69†	4.80 ± 1.47	4.89 ± 1.87	4.87 ± 0.81	24.72 ± 3.50†

*Data are expressed as % ± standard deviation, $n = 6$; †$p < 0.05$ between 37°C and 4°C (Student's t test). Tc-99m = Tc-99m (V) DMSA; γ = gamma-globulin; β = beta-globulin; α_2 = alpha-2-globulin; α_1 = alpha-1-globulin.
The bands in the agarose electrophoresis analysis:

Table 4. Affinity chromatography: percentage of Tc(V)-DMSA and Ga-citrate bound to transferrin*

	Bound to transferrin	
	37°C	4°C
Sample type		
Tc(V)-DMSA plasma	1.67 ± 0.82	1.83 ± 1.17
Tc(V)-DMSA alone	1.50 ± 0.84	1.50 ± 0.55
Ga-citrate plasma	87.50 ± 1.87†	95.50 ± 1.87†
Ga-citrate alone	1.83 ± 0.75	1.67 ± 0.52

*Data are expressed as % ± standard deviation, $n = 6$; †$p < 0.05$ between 37°C and 4°C (Student's t test).

Ga-67 alone, Tc(V)-DMSA plasma and Tc(V)-DMSA alone samples were applied to the affinity column, the retained radioactivity ranged between 1% and 2% at both 37°C and 4°C.

Temperature significantly affects plasma protein binding

Our results showed that there was a significant ($p < 0.05$) difference in the levels of plasma protein binding at 37°C and 4°C (Tables 2–4), suggesting that temperature significantly affects the plasma protein binding of these two radiopharmaceuticals.

Discussion

The interaction of a radiopharmaceutical with plasma proteins is an important characteristic influencing both its biodistribution over the whole body and its pharmacokinetics. In this study, we explored and compared the blood binding and transport of Tc(V)-DMSA and Ga-citrate because both radiotracers can be used to image tumors and inflammation. We found that these two radiotracers have similar and different properties.

In whole blood, both Tc(V)-DMSA and Ga-citrate were distributed primarily in the plasma and had a high degree of plasma protein binding. In addition, both radiotracers showed a significant difference in plasma protein binding between 37°C and 4°C, which corroborated the finding of Tsan et al [18] that the percentage of Ga-67 binding to plasma proteins was temperature dependent. This temperature effect is most likely due to a combination of increased dissociation of Ga-67 from Ga-67–plasma protein complexes and increased diffusion of Ga-67 at 37°C [18].

A similar conclusion can be drawn about the temperature effect of Tc(V)-DMSA–plasma protein binding.

An interesting finding of the present study was that different protein groups have distinctly different affinities for binding Tc(V)-DMSA and Ga-citrate: Tc(V)-DMSA bound mostly to the albumin fractions, while Ga-citrate bound mostly to β-globulins. Electrophoresis at 37°C showed greater dissociations of these two protein-bound radiopharmaceuticals compared with those at 4°C.

Our finding that albumin is the main transport protein of Tc(V)-DMSA may contribute to a better understanding of the biodistribution and pharmacokinetics of this radiotracer. Binding of Tc(V)-DMSA to albumin contrasts with the negligible albumin binding of the renal imaging agent Tc-99m DMSA (III-DMSA). III-DMSA binds preferably to α and β globulin fractions. This kind of bound form permits III-DMSA to reach specific target tissues, such as the kidneys, where it accumulates [19].

Maiorino et al [20] observed that DMSA, a therapeutically effective chelating agent for treating lead intoxication, was bound by disulfide linkage mostly to albumin in the plasma. Plasma proteins appear to serve as a depot and reservoir for DMSA. Some other radiopharmaceuticals bind with albumin, such as the tumor-imaging agent Tc-99m-DL-homocysteine [21]. Tc(V)-DMSA had a bi-exponential blood clearance and cumulative urine excretion in New Zealand white rabbits [12]. Wang et al [22] demonstrated the 18-kDa peptide expressed on tumor cells is a principal plasma albumin-binding protein (ABP). Albumin is the most abundant protein in plasma, present at 50 g/L, and has a half-life of 19 days in humans. Plasma albumin was found to bind specifically with the corresponding ABP, which is believed to play a significant role in transcytosis. Our results showed that albumin serves as a carrier for the transport of Tc(V)-DMSA, which may decrease the elimination of this radiotracer into urine, maintain its blood concentration, and subsequently cause it to accumulate in tumor cells or inflamed tissue. Further study is needed to investigate the mechanism of Tc(V)-DMSA release from Tc(V)-DMSA-albumin complexes and its uptake by tumor cells or inflamed tissue. Whether such a binding-releasing interaction between Tc(V)-DMSA and plasma albumin is a significant factor in its accumulation in tumor cells or inflamed tissue remains to be elucidated.

The present study, as well as other studies [16–18], demonstrated that the main plasma protein binding Ga-citrate is transferrin. It is transported to normal, inflamed or tumorous tissues predominantly in Ga-67–transferrin complexes. The entry of Ga-citrate into tumor tissue involves simple diffusion of the unbound or loosely bound form of Ga-citrate, whereas its uptake is strongly promoted by its binding to transferrin. Moreover, Ga-citrate localization in tumors involves endocytosis of Ga-67–transferrin receptor complexes via the transferrin receptor [17].

Both Tc(V)-DMSA and Ga-citrate, which are used to image inflammation and some tumors, bind primarily to plasma proteins. The major carrier of Tc(V)-DMSA is albumin and that of Ga-citrate is transferrin. These different binding proteins may affect their biodistribution and pharmacokinetics.

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Tc-99m (V) DMSA 與人類血漿蛋白質作用機轉之研究

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探討 Tc-99m (V) DMSA 於細胞內的作用機轉之前，先針對血液中的血漿與 Tc-99m (V) DMSA 之間的作用機制進行研究；並且以鎵 －67 進行相同的實驗進而加以比較。採用超過濾法、電泳法、膠過濾色層分析法及親合色層分析法進行實驗。於 37°C 時，Tc-99m (V) DMSA 有 45－54% 與血漿蛋白質結合；若於 4°C 時，則有 73－80% 與血漿蛋白質結合。至於鎵 －67 與血漿蛋白質結合個別為 43－53% (37°C) 及 75－81% (4°C)。於電泳，Tc-99m (V) DMSA 有 36.05 ± 2.48% (37°C) 為結合於白蛋白，至於 4°C 時，有 60.04 ± 1.87% 與白蛋白結合；至於鎵－67 則主要結合於乙型球蛋白 (37°C，34.23 ± 1.37%；4°C，55.71 ± 3.69%)。親合色層分析法發現鎵 －67 確實是結合於載鐵質，而 Tc-99m (V) DMSA 則否。此實驗證實 Tc-99m (V) DMSA 於血液中，主要與血漿蛋白質結合，其中大部分是與白蛋白加以結合。至於鎵 －67 則主要與乙型球蛋白中載鐵質加以結合。

關鍵詞：白蛋白，鎵－67，血漿蛋白質，Tc-99m (V) DMSA，載鐵質
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