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Lymphatic Delivery of ^{99m}Tc-labeled Dextran Acetate Particles Including Cyclosporine A

Kim, Jin[1], Kyong-Hwan Chung[2], Chang-Moon Lee[3], Young-Soon Seo[4], Ho-Chun Song[4], and Ki-Young Lee[2,5]*

[1]*Department of Advanced Chemicals Graduate School, Chonnam National University, Gwangju 500-757, Korea*
[2]*Center for Functional Nano Fine Chemicals, Chonnam National University, Gwangju 500-757, Korea*
[3]*Department of Nuclear Medicine and Research Institute of Clinical Medicine, Chonbuk National University School of Medicine, Jeonju 561-712, Korea*
[4]*Department of Nuclear Medicine, Chonnam National University Medical School, Gwangju 500-757, Korea*
[5]*Department of Applied Chemical Engineering and The Research Institute for Catalysis, Chonnam National University, Gwangju 500-757, Korea*

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Biodistribution and lymphoscintigraphy of cyclosporine A (CyA) and technetium-99m (^{99m}Tc) were studied using ^{99m}Tc-labeled dextran acetate (DxA) including CyA. DxA particles were prepared from dextran with acetic anhydride, and CyA was loaded into them. Lymphatic delivery of ^{99m}Tc-labeled DxA particles containing CyA was evaluated after subcutaneous injection into the foot pad of rats and compared with those of ^{99m}Tc-labeled human serum albumin (HSA). The labeling efficiency of CyA-loaded ^{99m}Tc-DxA particles was about 95% at 30 min. The labeling efficiency maintained stably above 80% for 12 h. The percent injected dose (%ID) of CyA-loaded ^{99m}Tc-DxA was similar to that of ^{99m}Tc-HSA at the inguinal lymph node after 40 min. The CyA-loaded ^{99m}Tc-DxA could be as well distributed as ^{99m}Tc-HSA through the lymph node. The DxA particles could steadily distribute the CyA as well as the ^{99m}Tc radiolabeling through the lymph node.

Keywords: Dextran acetate, lymphatic delivery, cyclosporine A, technetium-99m labeling, human serum albumin

CyA is one of several biologically active cyclosporines produced by the fungus *Tolypocladium inflatum* Gams or *Cylindrocapron lucidum* inflatum Booth [30]. It is an important immunosuppressive drug used to prevent allograft rejection in organ and tissue transplantation [7]. However, CyA has been restricted in use for clinical treatment owing to its low solubility and bioavailability [43]. The oral administration of CyA for a long time may also result in harmful reversible side effects such as hypochromic anemia, marrow hypoplasia, and lymphopenia [6].

Studies have been condncted to improve the therapeutic efficacy of CyA and to exclude its side effects. It was suggested that particulate formulations provide the better pharmacokinetic profiles and increased the oral bioavailability of the drugs [33, 35]. Microformulations [12, 16, 25, 38], complexation with cyclodextrin [31], and microspheres [8, 17, 39] increase the miscibility and oral bioavailability of CyA. Despite the great therapeutic interest of CyA, the bioavailability of oral administration is low with a high variability [4, 42] On the other hand, the lymphatic transport of therapeutic drugs has recently been developed to enhance the therapeutic efficacy with its immunosuppressive activity [20]. The lymphatic transport of CyA can induce a considerable improvement of bioavailability.

Dextran has excellent biocompatibility and has been used as a carrier for a variety of bioactive agents such as small molecule drugs, peptides, proteins, and enzymes [26, 36]. However, hydrophobic drugs, such as paclitaxel, precipitate out when they are directly loaded into dextran matrices due to its highly hydrophilic nature. The synthesis of an amphiphilic copolymer of dextran with poly(ε-caprolactone) (PCL) as side chains should combine properties of the hydrophilic dextran with hydrophobic PCL to produce a biocompatible copolymer with an enhanced capacity for solubilizing paclitaxel and capable of forming suitable film formulations for potential application in preventing postsurgical adhesions.

There has been considerable interest in developing dextran-based radiopharmaceuticals containing ^{99m}Tc, which rely on the dextran to stabilize the reduced ^{99m}Tc. ^{99m}Tc-dextran has been thoroughly evaluated as an angiocardiographic agent [9, 18], a lymphoscintigraphic agent [10, 11, 15, 19, 44], an inflammation imaging agent [4, 5], and a protein losing

*Corresponding author
Phone: 82-62-530-1843; Fax: 82-62-530-1819;
E-mail: kilee@chonnam.ac.kr

enteropathy imaging agent [2, 3, 24]. Lymphoscintigraphy is an established method for determining the lymphatic drainage patterns from malignant tissues and dematous extremities. ^{99m}Tc dextran has recently been used for sentinel lymph node biopsy in breast cancer [1, 35, 40], which is a surgical technique that avoids unnecessary axillary lymph node dissection [14, 33, 45].

^{99m}Tc-dextran, however, has some disadvantages, such as colloidal contamination and loss of labeling *in vivo*, due to the weak chelating ability of the dextran hydroxyl groups. To solve these problems, a modified dextran containing a cysteamine ligand system had been designed to chelate the ^{99m}Tc that would result in a more stable ^{99m}Tc-dextran complex. The dextran was modified by oxidizing with sodium periodate [21, 28], coupled to cysteamine, and was reduced to form amines to alter the hydroxyl group of dextran [29].

The HSA has been typically used as a radiopharmaceutical with ^{99m}Tc radiolabeling. ^{99m}Tc-HSA radioaerosols are readily available, are of good scintigraphic quality, and are inexpensive. There has been an increase in the use of ^{99m}Tc radioaerosols for clinical investigation [23]. However, it is impossible to transport therapeutic drugs in company with ^{99m}Tc labeling.

We are concerned with the preparation of a useful lymphatic carrier that can transport the CyA in company with ^{99m}Tc radiolabeling. In the present study, we prepared the DxA from dextran to use as a lymphatic transport carrier for CyA and ^{99m}Tc radiolabeling. CyA was loaded and ^{99m}Tc was labeled on the DxA particles. The biodistribution of ^{99m}Tc-labeled DxA particles with CyA loading by lymphatic delivery was investigated in rats. CyA release from DxA particles was measured at lymph node condition. The biodistributions of ^{99m}Tc labeled DxA particles containing CyA were compared with those of ^{99m}Tc-labeled HAS, employed as a control, by the radioactivity of the iliac lymph node and background radioactivity. We also investigated the efficiency and stability of ^{99m}Tc labeling on CyA-loaded ^{99m}Tc-DxA particles.

Materials and Methods

Reagents

Dextran (MW 60,000–90,000) was purchased from Hayashibara (Tokyo, Japan). HSA was donated from the Chonnam National University Hospital (Gwangju, Korea). CyA was donated from Chonggeundang (Seoul, Korea). Acetic anhydride, dimethylsulfoxide, dichloromethane, and pyridine were purchased from Sigma (St. Louis, U.S.A.). All other chemicals were of reagent grade and used without further purification.

Synthesis of DxA

DxA was synthesized according to the Motozato *et al.* method [32]. Dextran (6 g) was suspended in 60 ml of formamide and dissolved with vigorous stirring at 54°C. Pyridine (18 ml) and acetic anhydride (40 ml) were added into the solution. The mixture was incubated under stirring at 54°C for 48 h. DxA was precipitated by addition of 500 ml of water, and washed with water and methanol several times. The final product was obtained *via* freeze drying after dialysis against water. The introduction of acetyl moieties to dextran was confirmed by Fourier transform infrared (FT-IR; Nicolet, Magna IR 50) analysis.

Preparation of CyA-loaded DxA Particles

DxA (40 mg) and CyA (20 mg) were dissolved in 10 ml of dimethylsulfoxide loaded into a dialysis tube that cuts off large molecules above 12,000 g/mol. The mixture in the dialysis tube was dialyzed in distilled water every 3 h for a day. Finally, the DxA particles were obtained by freeze drying.

Characterization of DxA

The formation of DxA was confirmed by Fourier transform infrared (FT-IR; Nicolet, Magna IR 50) analysis. The morphologies of dextran, DxA, and CyA-loaded DxA particles were obserbved using the scanning electron microscope (SEM; JEOL, JSM 5400, Japan). Their particle size distributions were measured by a particle size analyzer (PSA; Malvern Instruments Inc., U.S.A.). Thermogravimetric analysis (TGA; Mettler, U.S.A.) was performed on the dextran and DxA particles. The kinematic viscosities of dextran and DxA solution (1 wt%) were measured as the time required for a volume of liquid to flow under gravity through a Cannon-Fenske style glass capillary tube at 25°C.

Drug Release of the CyA-Loaded DxA Particles

The DxA particles loading CyA were introduced in the tube with phosphate buffer saline (PBS) solution adjusted to the normal human blood condition (pH 7.4). CyA release from the CyA-loaded DxA particles was carried out in a culture medium at 37°C. The release amount of CyA was measured using high-performance liquid chromatography (HPLC; M-510; Waters, Japan).

Radiolabeling and Stability Test

CyA-loaded DxA particles suspended in PBS (2 ml) were added to a vacuum vial. Then, ^{99m}Tc-pertechnetate (75 mCi/2 ml) was added to the vial containing the reducing agent, stannous chloride (Amersham Plc., Buckinghamshire, U.K.). The mixture of ^{99m}Tc-pertechnetate and stannous chloride was added to the vacuum vial containing the CyA-loaded DxA particles suspension. They were allowed to bind for 20 min at room temperature.

The labeling efficiencies were assessed by instant thin-layer chromatography (ITLC; AR-2000 scanner; Bioscan Inc. Washington DC, U.S.A.) using silica gel coated fiber sheets. The ITLC analysis was performed using saline and acetate as mobile phase. Free technetium moved to the top of the paper for ITLC and the radiolabeled DxA particles stayed at the application point. HSA kits (Daiichi Radioisotope Labs. Ltd, Tokyo, Japan) were labeled with ^{99m}Tc and quality assured according to the manufacturer's indications. Labeling efficiency was checked using the following equation, as described in previous literature [27]:

$$\text{Labeling efficiency (\%)} = [(\text{total radioactivity of } {}^{99m}\text{Tc} - \text{radioactivity of } {}^{99m}\text{TcO}_4^-)/\text{total radioactivity of } {}^{99m}\text{Tc}] \times 100$$

The stability of CyA-loaded ^{99m}Tc-DxA was evaluated at room temperature for 12 h. The bound and free forms of ^{99m}Tc were determined using ITLC and 5% bovine serum albumin (BSA)-impregnated paper chromatography/saline.

Imaging Studies in Rats

Lymphoscintigraphy was performed with 10 outbred female Sprague-Dawley (SD) rats (Samtako Bio, Osan, Korea; 16 week-old, ~250 g).

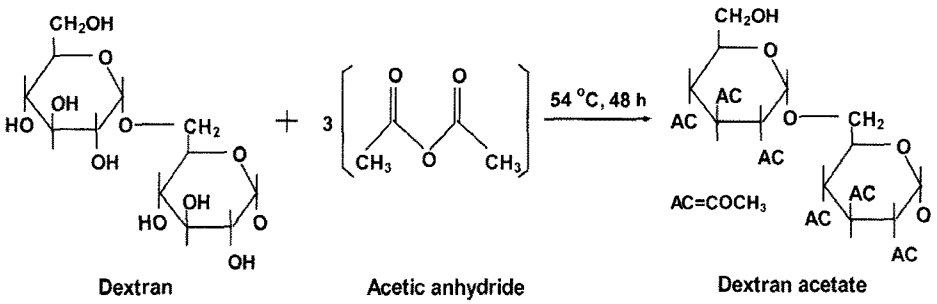

Fig. 1. Synthesis of DxA from dextran with acetic anhydride.

Thereafter, 9.25 MBq (0.25 mCi/0.1 ml) of CyA-loaded ^{99m}Tc- DxA was subcutaneously injected into the foot pad of each rat under ketamine anesthesia (200 mg/kg i.p.). ^{99m}Tc-HSA was also applied to rats to compare with the biodistribution of CyA-loaded ^{99m}Tc- DxA. Planar images were obtained at 10 min, 40 min, and 70 min using a gamma camera equipped with a 6-mm pin-hole collimator (DST-XLi; GE Healthcare, U.S.A.). A total of 500 k counts were obtained for each image. The radioactivity of the iliac lymph node and background radioactivity (abdominal cavity) were measured on the VISION POWERstation software, and the tissue-to-background ratio (TBR) was assessed as follows:

TBR=Radioactivity of iliac lymph nodes/Background in abdomen

RESULTS AND DISCUSSION

Formation of DxA

The DxA was formed from the reaction of substitution of the hydroxyl group in dextran to acetic anhydride according to the reaction path shown in Fig. 1. FT-IR spectra of dextran and DxA are presented in Fig. 2. In the infrared vibrate bands of DxA, the C=O combination at 1,747 cm^{-1}, CH_3 band at 1,431 cm^{-1}, and O-C=O combination band at 604 cm^{-1} appeared newly in comparision with those of dextran. The OH band at 3,450 cm^{-1} was abruptly diminished in FT-IR spectra of DxA particles. These indicate that the hydroxyl groups of dextran were substituted to $COCH_3^-$ groups by the reaction. These prove that the DxA is formed by the reaction of dextran with acetic anhydride.

Particle size distributions of dextran and DxA are shown in Fig. 3. The average particle size of dextran was about

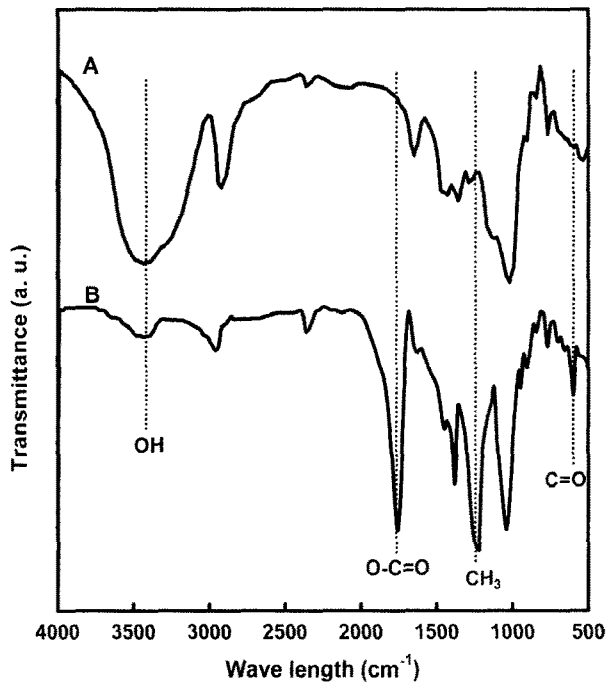

Fig. 2. FT-IR spectra of (A) dextran and (B) DxA.

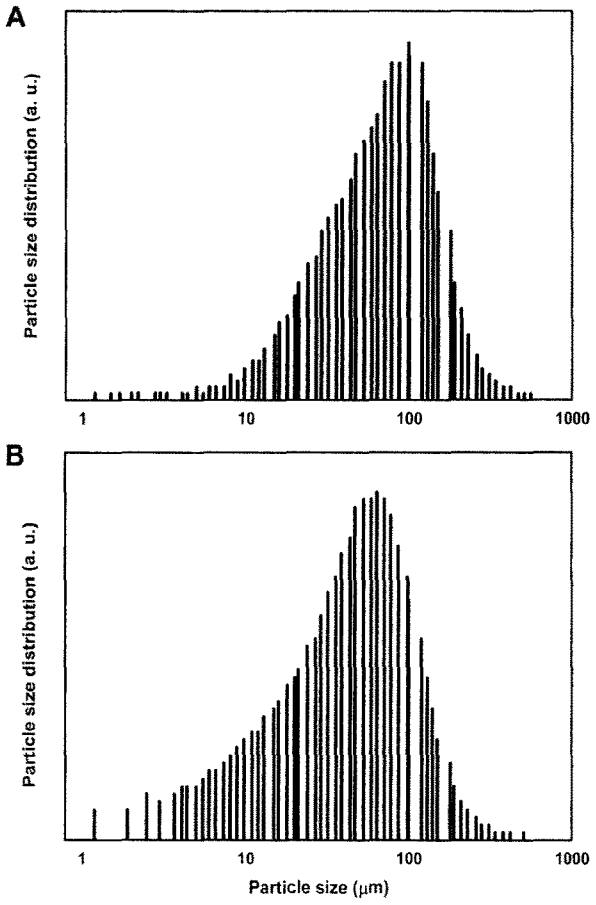

Fig. 3. Particle size distribution of (**A**) dextran and (**B**) DxA particles.

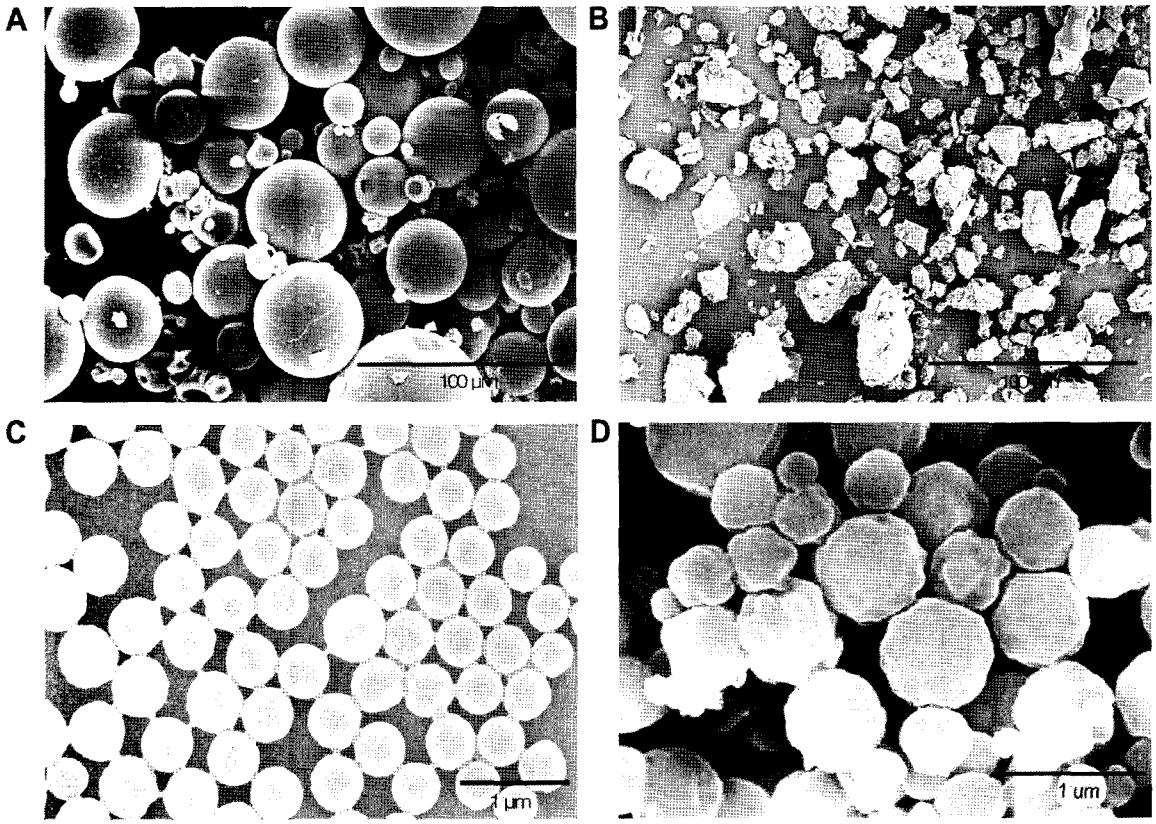

Fig. 4. SEM photographs of (**A**) dextran, (**B**) DxA, (**C**) CyA-loaded DxA particles after dialysis, and (**D**) CyA-loaded DxA particles after freeze drying.

71 μm. The particle size was reduced to 45 μm after converting to DxA. SEM photos of dextran DxA, and CyA-loaded DxA particles are shown in Fig. 4. The morphology of dextran was a regular spherical shape, as presented in Fig. 4A. The morphology of DxA was an amorphous shape and they were aggregated closely after freeze drying. Cracked particles were observed after grinding of the freeze-dried DxA particles, as shown in Fig. 4B.

The SEM photograph of CyA-loaded DxA particles after dialysis is shown in Fig. 4C. The morphology of CyA-loaded DxA was also sphere type. The microspheres were connected with the small rods between the particles. The connection rods between every CyA-loaded DxA particle disappeared after freeze drying (Fig. 4D). The morphology of CyA-loaded DxA particles after drying was crumpled at their surface. The particle size of the CyA-loaded DxA particles was reduced slightly with drying. The particle size of CyA-loaded DxA was about 500 nm.

The melting temperatures of dextran and DxA estimated from TGA results were about 345°C and 410°C, respectively. The value of DxA was higher than that of dextran. It seems that the molecular structure became stronger as the hydroxyl group was substituted to the acetic anhydride. Kinematic viscosities of dextran and DxA were measured to compare their fluidity. The kinematic viscosities of dextran and DxA solutions were 1.6 mm^2/s and 1.4 mm^2/s, respectively. This means that the drug delivery properties of the DxA solution would be superior to that of dextran solution.

CyA Release from DxA Particles

Fig. 5 shows profiles of CyA the releasing from DxA particles in normal human blood condition. The releasing conditions were adjusted to pH 7.4 by PBS solution. The CyA was released 30.3±1.5% after 12 h from the DxA particles. It has been reported that the extent of lymphatic transport of

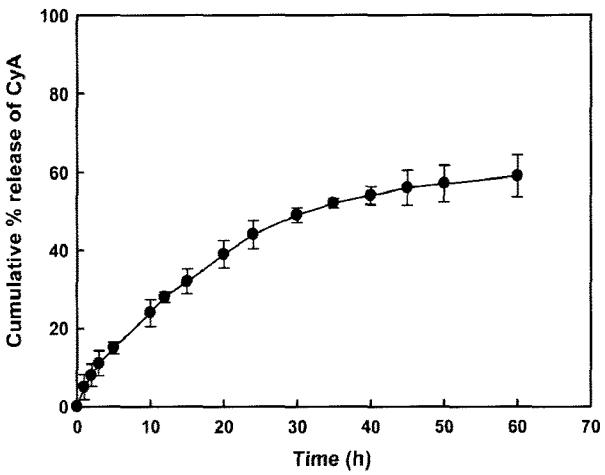

Fig. 5. CyA release from DxA particles.

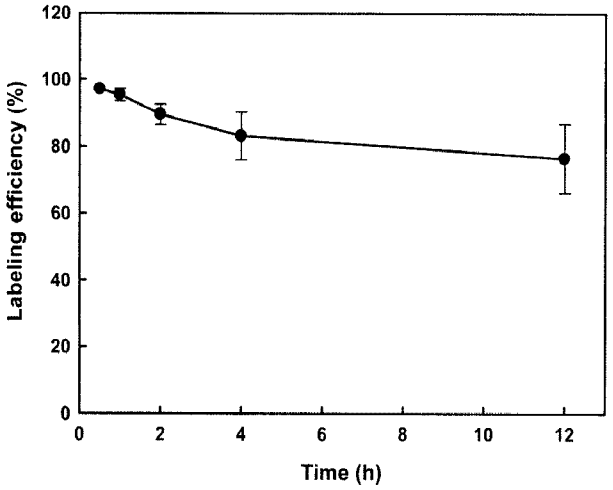

Fig. 6. Labeling efficiency of ^{99m}Tc-DxA particles.

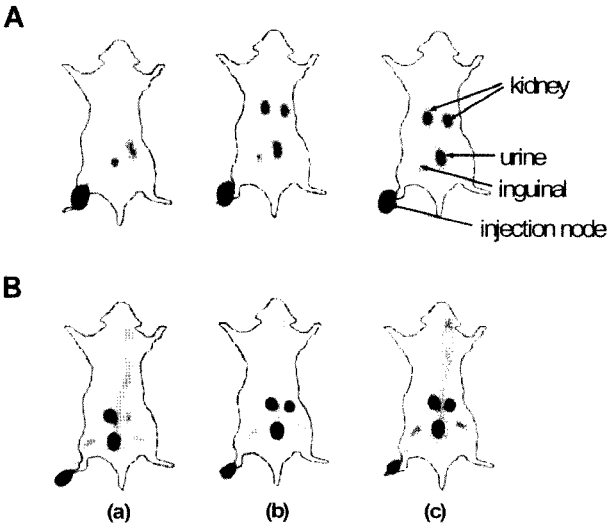

Fig. 7. Sequential scintigrams of rats obtained with subcutaneous injection of (**A**) CyA-loaded ^{99m}Tc-DxA or (**B**) ^{99m}Tc-HSA at (a) 10 min, (b) 40 min, and (c) 70 min.

halotantrine after 12 h was 27.4±1.3% in analysis of cumulative % of halotantrine [20]. The release rate of CyA exhibited a similar value to that of halotantrine. CyA is such a poorly soluble drug that aggregation will quickly occur, which causes the decrease in dispersion degree and relatively poor absorption of CyA [40]. It has been known that the dose amount of CyA should be controlled to one-third of the oral dose amount when CyA is delivered by intravenous administration [13]. It resulted that DxA could be used as a drug carrier for CyA because it has a sufficient loading and release ability for CyA.

^{99m}Tc Labeling Efficiency and Stability

Fig. 6 shows the labeling efficiency of CyA-loaded ^{99m}Tc-DxA particles estimated by the radioactivity of ^{99m}Tc. The ^{99m}Tc labeling efficiency of CyA-loaded ^{99m}Tc-DxA particles was above 80% during the whole test period. The ^{99m}Tc labeling efficiency of the DxA particles exceeded 90% at 2 h during the process time. The result was similar to that of pulluran acetate microparticles in a previous study [27]. The high labeling efficiency maintained stably for 12 h. The labeling efficiency was superior to that of chitosan microparticles and maintained longer duration in the literature [22]. The labeling efficiency of ^{99m}Tc-HSA was reported as 95% at 30 min after reconstitution. The value of ^{99m}Tc-DxA particles was also about 95% at the same time in our results. It means that the DxA particles have a high labeling efficiency for ^{99m}Tc and a stable labeling durability equivalent to HSA. It indicates that the DxA particles can be used a radiopharmaceutical in substitution of HSA aerosols. In addition, DxA has the ability of drug delivery by lymph node, different to HSA aerosols. Therefore, it is expected that DxA will be able to load therapeutic drugs in company with ^{99m}Tc labeling.

Biodistribution of the CyA-Loaded ^{99m}Tc-DxA Particles

The mean dose amounts of CyA-loaded ^{99m}Tc-DxA and ^{99m}Tc-HSA labeling materials to the 5 rats were calculated from dosimetry of radioisotope before and after injection of the labeling materials. The mean dose amount of the labeling materials was determined as 0.25 mCi with injection of both CyA-loaded ^{99m}Tc-DxA and ^{99m}Tc-HSA.

Sequential scintigrams of rats after subcutaneous injection with CyA-loaded ^{99m}Tc-DxA particles and ^{99m}Tc-HSA are shown in Fig. 7. The radioactivities in the rats with injection of CyA-loaded ^{99m}Tc-DxA and ^{99m}Tc-HSA were examined at the organs of the rats. The scintigraphic images of the rats with CyA-loaded ^{99m}Tc-HSA showed radioactivity at the inguinal, urine, and kidney from 10 min after the injection. The scintigraphic image at axillary was not observed distinctively for injection of CyA-loaded ^{99m}Tc-DxA and ^{99m}Tc-HSA. The sequential scintigrams of the CyA-loaded ^{99m}Tc-DxA animals showed a similar trend with those of ^{99m}Tc-HSA. It means that the CyA-loaded ^{99m}Tc-DxA can be as well distributed as ^{99m}Tc-HSA through the lymph node.

Fig. 8 shows the %ID with delivering time at the iliac, popliteal, and axillary for the CyA-loaded ^{99m}Tc-DxA particles and ^{99m}Tc-HSA, respectively. The %ID of CyA-loaded ^{99m}Tc-DxA at the iliac was higher than that of ^{99m}Tc-HSA. At the popliteal and axillary, however, the %IDs exhibited a reverse trend compared with the results at the iliac. This trend accorded with the results at 40 min. It indicates that the distribution rate of ^{99m}Tc-HSA may be faster than that of CyA-loaded ^{99m}Tc-DxA. Although the distribution rate of CyA-loaded ^{99m}Tc-DxA was slow compared with that of ^{99m}Tc-HSA, the %ID of CyA-loaded ^{99m}Tc-DxA was much more than that of ^{99m}Tc-HSA at the axillary after 70 min. In contrast the %ID of ^{99m}Tc-HSA at the axillary decreased with processing time. The results indicate that the DxA can steadily distribute the CyA as

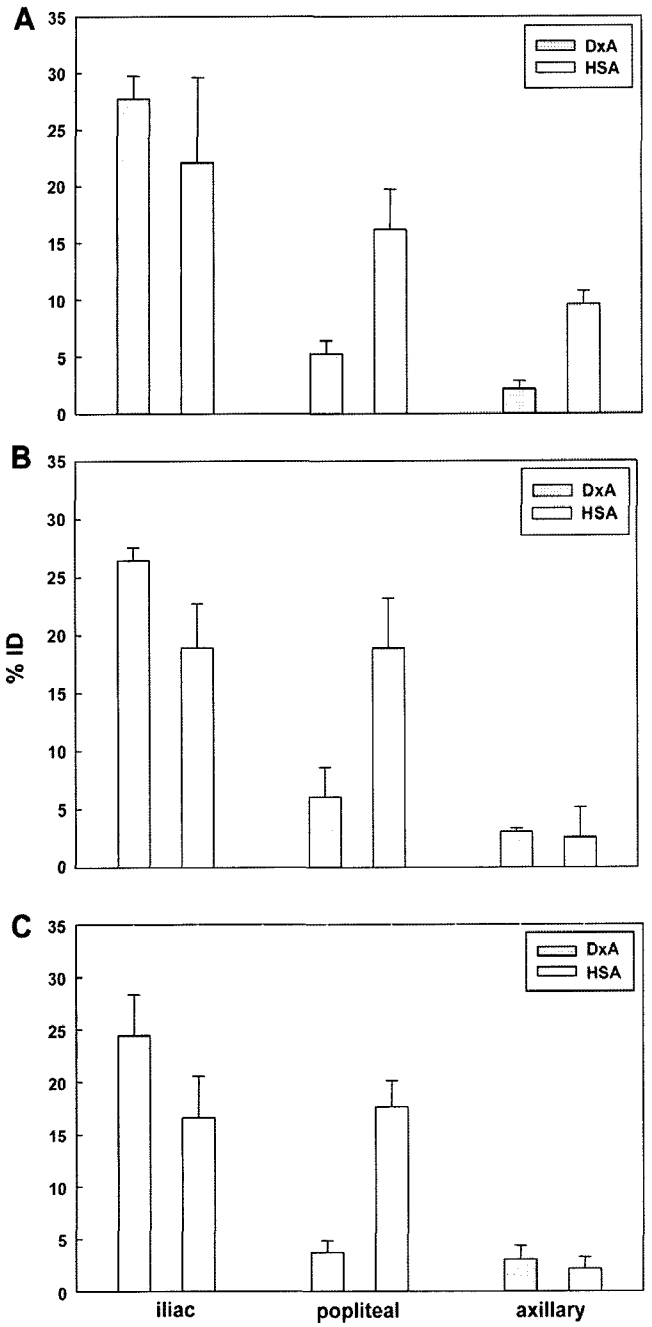

Fig. 8. Biodistributions in rats after subcutaneous injection of CyA-loaded ^{99m}Tc-DxA and ^{99m}Tc-HSA at (**A**) 10 min, (**B**) 40 min, and (**C**) 70 min.

well as stably maintain the ^{99m}Tc radiolabeling to the axillary through the lymph node.

In conclusion, the sequential scintigrams of the CyA-loaded ^{99m}Tc-DxA exhibited a similar trend with those of ^{99m}Tc-HSA. It means that the CyA-loaded ^{99m}Tc-DxA can be as well distributed as ^{99m}Tc-HSA through the lymph node. The %ID of CyA-loaded ^{99m}Tc-DxA was similar to that of ^{99m}Tc-HSA at the inguinal lymph node. It implied that the DxA particles can steadily distribute the CyA as well as the ^{99m}Tc radiolabeling through the lymph node.

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