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Basic evaluation of ^{67}Ga labeled digoxin derivative as a metal-labeled bifunctional radiopharmaceutical

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To develop metal-labeled digoxin radiopharmaceuticals with affinity with anti-digoxin antibody as well as Na^+,K^+-ATPase, a digoxin derivative conjugated with deferoxamine was synthesized. The derivative had a high binding affinity with ^{67}Ga at deferoxamine introduced to the terminal sugar ring of digoxin. The ^{67}Ga labeled digoxin derivative showed enough *in vitro* binding affinity and selectivity to anti-digoxin antibody as well as Na^+,K^+-ATPase. The ^{67}Ga labeled digoxin derivative is considered to be a potential metal-labeled bifunctional radiopharmaceutical for digoxin RIA as well as myocardial Na^+,K^+-ATPase imaging.

Key words: digoxin, radiopharmaceutical, deferoxamine, radioimmunoassay, Na^+,K^+-ATPase

INTRODUCTION

THE DEVELOPMENT of metallic radionuclide-labeled bifunctional radiopharmaceuticals with high affinity with and specificity for corresponding antibodies or receptors has long been desired in nuclear medicine. Short-lived radioactive metals, such as ^{67}Ga, ^{99m}Tc, and ^{111}In, have numerous advantages in the design of radiopharmaceuticals over ^{125}I, because of their superior specific activity, simplicity of labeling kit formation, and availability at reasonable cost. So various trials have been performed in this field, and are still progressing.

Recently we reported the potential usefulness of ^{123}I-labeled digoxin radiopharmaceutical for myocardial Na^+,K^+-ATPase imaging.[1] That study suggested that the first and second sugars at the C-3 position of digoxin, as well as the steroid aglycone, were essential for myocardial accumulation, but the third sugar could be a site capable of modifications for radiolabeling. These findings introduced a new approach for the design of digoxin-based bifunctional radiopharmaceuticals. That is to say, the introduction of a metal-chelating site to the third sugar residue of digoxin may have less effect on the binding ability and specificity of digoxin for the anti-digoxin antibody and/or Na^+,K^+-ATPase.

In the present study, we selected the ^{67}Ga-deferoxamine (^{67}Ga-DFO) system[2] as a metallic radionuclide-bifunctional chelating agent system and synthesized digoxin-deferoxamine[bis(o-carboxymethyloxime)] (DFO-digoxin conjugate). Its ^{67}Ga-labeling with high specific activity and *in-vitro* binding assay to the anti-digoxin antibody as well as Na^+,K^+-ATPase were performed. A biodistribution study in guinea pigs was then attempted in comparison with ^{67}Ga-DFO and ^{67}Ga-citrate.

MATERIALS AND METHODS

Digoxin and desferal (deferoxamine mesylate) were obtained from Aldrich (Milwaukee, WI), Ciba Geigy (Basel, Switzerland), respectively. Iron-free HCl was purchased from Nacalai Tesque (Kyoto, Japan). ^{67}Ga-citrate was obtained from Nihon Mediphysics (Takarazuka, Japan). "Amerlex digoxin", digoxin radioimmunoassay (RIA) kit, was obtained from

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Amersham (Buckinghamshire, England). All other reagents and solvents were of reagent grade.

The radioactivity of ^{67}Ga and ^{125}I was measured with an autogamma counter (ARC300, Aloka, Japan). Analytical thin-layer chromatography (TLC) was performed with Merk TLC plates (silica gel 60 F254 pre-coated, 0.25 mm layer thickness). The TLC plate was stained with iodine vapor to visualize spots on the plate. HPLC was performed in a Shimadzu LC-5A high performance liquid chromatograph (Shimadzu, Japan) equipped with a UV detector and a reverse-phase column (cosmosil 5 C 18, 8×250 mm; Nacalai Tesque, Japan). ^1H NMR at 300 MHz was performed with a Bruker AC-300 apparatus for DFO-digoxin conjugate, dissolved in dimethyl sulfoxide-D_6. Tetramethylsilane was used as the internal standard.

Chemical synthesis

N-hydroxysuccinimide ester of digoxin[bis(o-carboxymethyloxime)]. The synthetic reaction sequence from digoxin dialdehyde to N-hydroxysuccinimide ester of digoxin[bis(o-carboxymethyloxime)] was followed according to the method reported previously in detail.[3,4] In brief, as shown in Fig. 1, the terminal digitoxose in digoxin was cleaved to give digoxin dialdehyde by using sodium metaperiodate. The condensation reaction of digoxin dialdehyde and carboxymethoxylamine hemihydrochloride proceeded rapidly in sodium acetate/ethanol and a quantitative yield of digoxin[bis(o-carboxymethyloxime)] was obtained. The digoxin-dioxime derivative was immediately reacted with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide to give N-hydroxysuccinimide ester of digoxin[bis(o-carboxymethyloxime)].

DFO-digoxin conjugate. A mixture of desferal (656 mg, 1 mmol) and N-hydroxysuccinimide ester of digoxin[bis(o-carboxymethyloxime)] in 60 m*l* of dry pyridine was stirred and heated at 95–105°C for 36 hr under nitrogen. The reaction was monitored by TLC in chloroform/methanol/water (80/25/3 by volume); Rf 0.75=dioxime active ester, 0.43=N-hydroxysuccinimide, 0.26–0.37=DFO-digoxin conjugate, 0.12=DFO. The reaction mixture was evaporated in a high vacuum. Pure DFO-digoxin conjugate was isolated from the crude reaction mixture by HPLC, eluted with methanol and distilled water (6/4 by volume) at a flow rate of 1.4 m*l*/min with deferoxamine mesylate and DFO-digoxin conjugate which were eluted at 6.5 and 61.2 min, respectively. DFO-digoxin conjugate was obtained as a white powder (22% yield) and had a melting point of 167–169°C. ^1H NMR (δ, ppm): 0.65 (singlet, 3H, 18-CH_3), 0.85 (singlet, 3H, 19-CH_3), 1.02–1.92 (complex multiplet), 1.97 (singlet, 6H, terminal methyl group in DFO), 2.90–4.97 (complex multiplet), 5.95 (singlet, 1H, lactone, C=CH). Elemental anal. Calcd for $C_{95}H_{160}O_{32}N_{14}\cdot 3H_2O$: C, 55.27; H, 8.10; N, 9.50. Found: C, 55.12; H, 7.92; N, 9.63.

Determination of the conjugation ratio of DFO-digoxin conjugate

To test the progress of DFO-digoxin conjugate formation, a solution of the conjugate (Chelating ligand: 316 μM, DMSO) was mixed with a Fe^{3+} solution (Metallic ion: 316 μM of $FeNO_3$, at pH 3) in various ratios (Metal/Metal+Ligand=0, 0.2, 0.4, 0.6, 0.7, 0.8, 1.0 by volume), and the concentrations of the Fe complex were measured after 3 min with a spectrophotometer (330s, Hitachi, Japan). The amount of deferoxamine bidning to DFO-digoxin conjugate was determined by Job's method of continuous variation.[5]

Antigenicity of DFO-digoxin conjugate

Antigenicity of the newly synthesized digoxin derivative was determined with a commercially available digoxin RIA kit. Fifty microliters of DFO-digoxin conjugate solution {0 M (control), 0.219 nM, 2.19 nM, 21.9 nM, 219 nM, 2.19 μM; 1% BSA/saline}, 200 μl of ^{125}I labeled digoxin derivative solution, and 200 μl of anti-digoxin antibody coated beads were mixed and incubated for 30 min at 37°C. After centrifugation (1500×G, 15 min), the supernatant was decanted. The ^{125}I radioactivity bound to the beads was measured and the ratios of binding counts to total counts were calculated. The amount of digoxin in the DFO-digoxin conjugate sample was estimated from the standard curve by simple extrapolation and the equivalent of digoxin in DFO-digoxin conjugate was calculated.

Radiolabeling of DFO-digoxin conjugate

The ^{67}Ga solution was purified as reported by Furukawa et al.[6] with some modifications in regard to the final extraction of ^{67}Ga as ^{67}Ga-citrate instead of ^{67}Ga-chloride by the method introduced by Horiuchi et al. (unpublished). Briefly, ^{67}Ga-citrate (292 MBq, 584 MBq/m*l*), 0.1 M ascorbic acid, and concentrated HBr were mixed at a volume ratio of 4: 1: 7 and extracted with the same volume of butyl acetate. After evaporation of the organic solvent, the clear residue was extracted with a solution containig 100 μl of 0.1 N iron-free HCl, 25 μl of 0.1 M ascorbic acid, and 175 μl of concentrated HBr. The solution was then extracted with 500 μl of butyl acetate, separated, and evaporated in the same way as above. Finally, 50 μl of sodium citrate (2 mg/m*l*) was added to the clear residue.

The radiolabeling of DFO-digoxin conjugate was carried out by adding 50 μl of the purified high

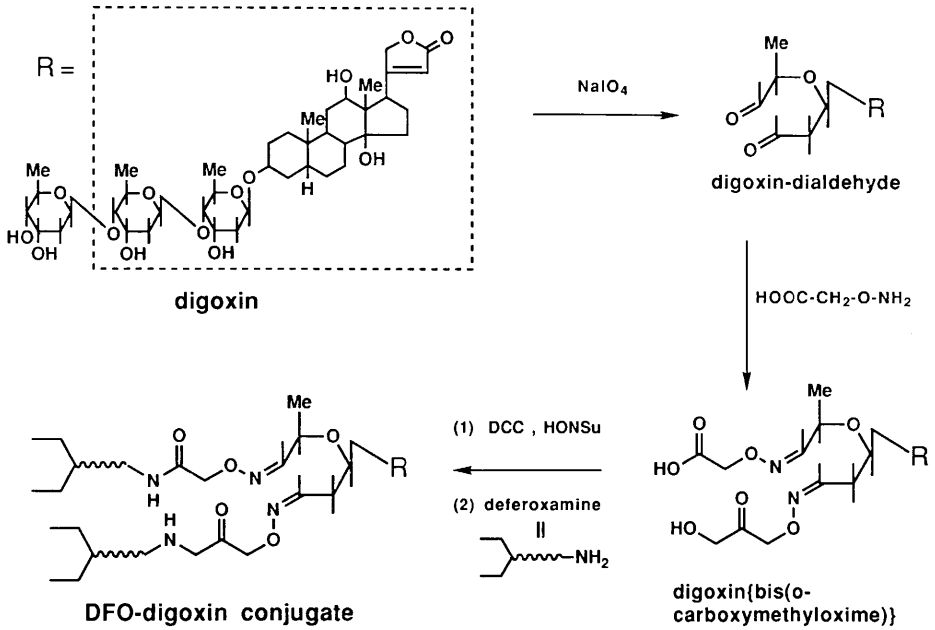

Fig. 1 The synthetic reaction sequence from digoxin to DFO-digoxin conjugate.

specific activity ^{67}Ga solution along with 50 μl of the previously prepared DFO-digoxin conjugate (50 μg/m*l*, DMSO). The addition was followed by simple mixing and standing for 1 hr. The labeling efficiency was analyzed by cellulose acetate electrophoresis (EP) in veronal buffer (I=0.05, pH 8.6) with a stationary current of 0.8 mA/cm for 40 min. The labeling efficiency percentage was estimated as the ratio of the radioactivity associated with the DFO-digoxin conjugate to the total radioactivity in each strip. As a control, both the ^{67}Ga-citrate solution and the ^{67}Ga-DFO were also analyzed under the same conditions. The radiolabeled ^{67}Ga-DFO-digoxin conjugate was diluted 40,000 times with 1% BSA/saline solution for use in the RIA.

RIA using ^{67}Ga labeled DFO-digoxin conjugate

The RIA with ^{67}Ga-DFO-digoxin conjugate (^{67}Ga RIA) was adapted from the ^{125}I RIA, using ^{67}Ga-DFO-digoxin conjugate instead of ^{125}I labeled digoxin derivate. The standard digoxin (0.35–5.0 ng/m*l*) used was provided by a commercial RIA kit.

The cross-reactivity of ^{67}Ga RIA with the DFO-digoxin conjugate to ouabain (1 ng–10 μg/m*l* saline) was analyzed following the above manufacturer's instructions, but using ouabain solution instead of standard digoxin solution.

In-vitro *Na^+,K^+-ATPase binding studies*

The effect of ouabain on the binding of ^{67}Ga-DFO-digoxin conjugate to Na^+,K^+-ATPase known as digitalis receptor, was studied according to a method described previously,[1,7] using the crude Na^+,K^+-ATPase fraction separated from guinea-pig kidney cortex.[8] As a reference, ^{3}H-digoxin binding was also determined.

Biodistribution studies

Male guinea pigs weighing 300 g were injected with ^{67}Ga-DFO-digoxin conjugate, ^{67}Ga-DFO, or ^{67}Ga-citrate (200 μl, 131 KBq) via the femoral vein and then killed by cervical decapitation at 60 min after the injection. Tissue accumulation was calculated as the percentage injected dose/gram of tissue.

RESULTS

Chemical synthesis

The synthetic sequence is outlined in Fig. 1. Isolated DFO-digoxin conjugate was characterized by ^{1}H NMR measurement and elemental analysis. From these data, it was demonstrated that DFO-digoxin conjugate had two deferoxamine molecules attached (Fig. 2). As a solid, DFO-digoxin conjugate was stable at 4°C. In solution, the conjugate was stable in methanol and water (6/4 by volume) for several days.

Determination of the conjugation ratio of DFO-digoxin conjugate

It is well known that desferal (or deferoxamine) has high binding affinity with Fe^{3+} (log K=30.6) and Ga^{3+}(log K=28.0). Figure 3 shows the line graph obtained by plotting absorbance against the ratio of metal concentration to metal plus ligand concentration. The maximum occurred at a ratio of about

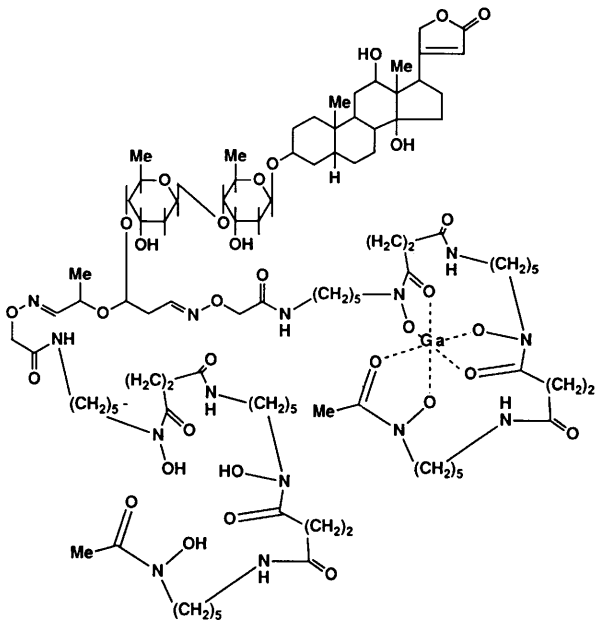

Fig. 2 The structure of ^{67}Ga labeled DFO-digoxin conjugate.

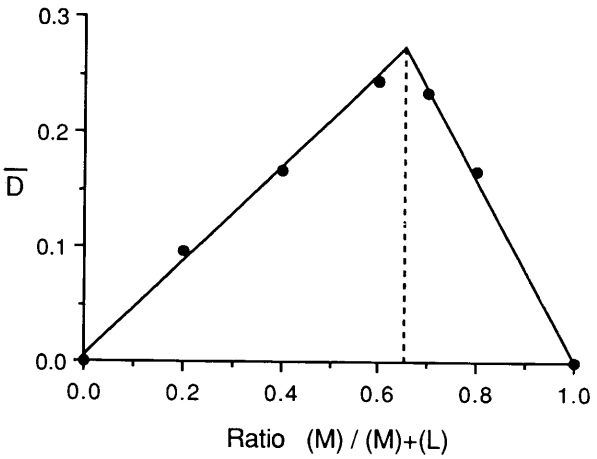

Fig. 3 Graphical determination of composition of Fe-DFO-digoxin conjugate chelate: D is the optical density difference at 460 nm.

0.66, indicating that Fe^{3+} and DFO-digoxin conjugate made a complex with Fe^{3+}/ligand ratio of 2/1. This showed that there were two binding sites for Fe^{3+} in DFO-digoxin conjugate.

Antigenicity of DFO-digoxin conjugate

The antigenicity of the newly synthesized DFO-digoxin conjugate was then determined. Table 1 shows that as the amount of the DFO-digoxin conjugate increased, a clear decrease in the radioiodinated binding fraction was detected. The immunoactivity was the same for 2.19 nmol of DFO-digoxin conjugate and 2.05 nmol of digoxin.

Radiolabeling of DFO-digoxin conjugate

The specific activity of the purified gallium solution used for the radiolabeling was 4.4 GBq/m*l*. DFO-digoxin conjugate was easily radiolabeled with the purified iron-free ^{67}Ga extracted as ^{67}Ga-citrate by a simple mixing reaction. The average labeling yield was 90% (85–94%) calculated after electrophoretic analysis (Fig. 4) and the specific activity of ^{67}Ga-DFO-digoxin conjugate reached 79 GBq/mg (159 MBq/nmol). Electrophoretic analysis did not detect the presence of free ^{67}Ga-DFO and ^{67}Ga-citrate in the reaction mixture. ^{67}Ga-DFO-digoxin conjugate diluted 10^2 times was chemically stable in BSA solution within one half-life of the radionuclide.

RIA using ^{67}Ga labeled DFO-digoxin conjugate

The digoxin RIA was performed with 200 μl per sample of ^{67}Ga-DFO-digoxin conjugate, providing approximately 20,000 counts per minute (cpm) per tube as described under MATERIALS AND METHODS. The ^{67}Ga RIA gave a good linearity between the percent bound/total counts (% B/T) and the concentration of digoxin (Fig. 5). Figure 5 shows the specificity of ^{67}Ga RIA to ouabain. The cross-reactivity to ouabain was below 0.1% of the digoxin binding, and was as low as in ^{125}I RIA (below 1.0%).

In-vitro *Na^+,K^+-ATPase binding studies*

Based on the antigenicity of DFO-digoxin conjugate

Table 1 The effect of the concentration of DFO-digoxin conjugate on the binding of ^{125}I labeled digoxin derivative to the anti-digoxin antibody

	Dilution of DFO-digoxin conjugate solution					
	original concentration of DFO-digoxin conjugate : 2.19 μM					
	original	$\times10^{-1}$	$\times10^{-2}$	$\times10^{-3}$	$\times10^{-4}$	control (0 M)
Bound / Total (%)	1.6	2.3	8.4	23.9	49.6	52.8
digoxin concentration *				2.05 nM		

* caluculated from the standard curve of ^{125}I digoxin RIA kit

to anti-digoxin antibody, the *in-vitro* binding to Na^+,K^+-ATPase was compared with that of ^3H-digoxin (Fig. 6). Studies with ouabain showed that the IC_{50} value for the binding of ^{67}Ga-DFO-digoxin conjugate to Na^+,K^+-ATPase was very similar to that of ^3H-digoxin.

Biodistribution studies

Table 2 shows the biodistribution of ^{67}Ga-DFO-digoxin conjugate, ^{67}Ga-DFO, and ^{67}Ga-citrate in guinea pigs. ^{67}Ga-DFO-digoxin conjugate showed higher myocardial accumulation than ^{67}Ga-DFO or ^{67}Ga-citrate.

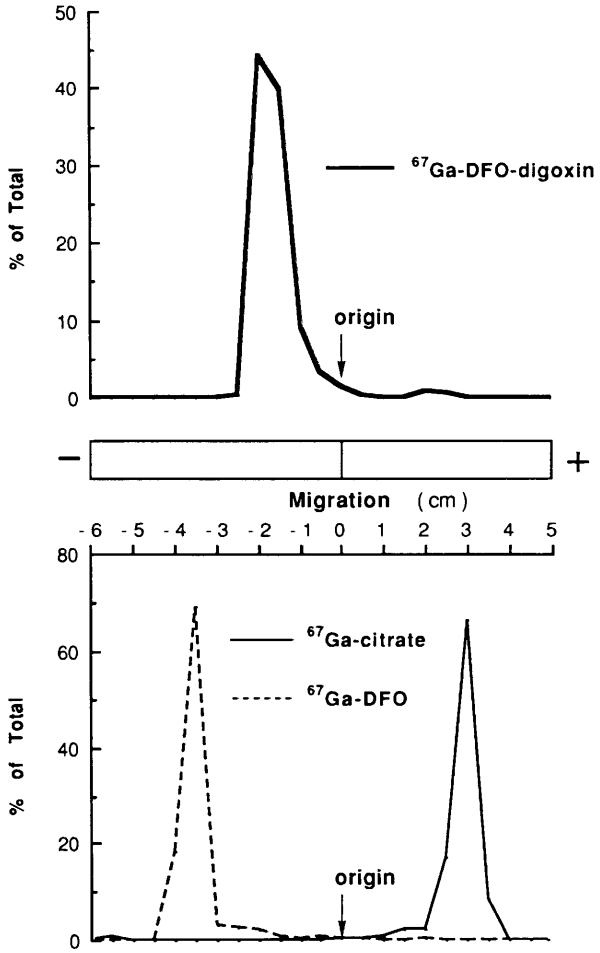

Fig. 4 Electrophoretic analysis of ^{67}Ga-DFO-digoxin conjugate, ^{67}Ga-citrate and ^{67}Ga-DFO.

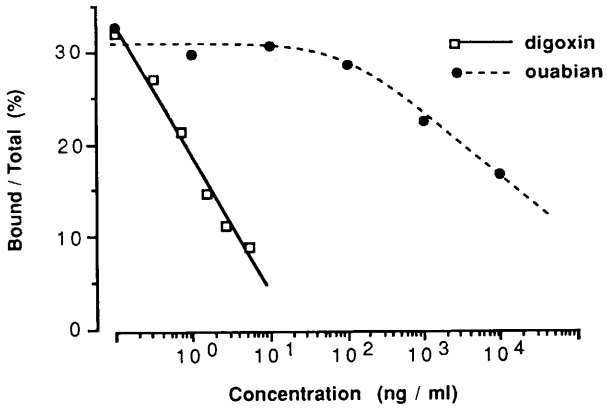

Fig. 5 The standard curve of ^{67}Ga digoxin-RIA and the cross-reactivity to ouabain.

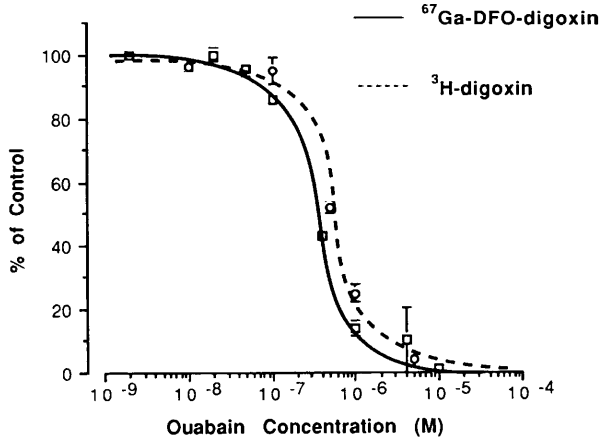

Fig. 6 Inhibition of ^3H-digoxin or ^{67}Ga-DFO-digoxin conjugate binding to guinea-pig kidney Na^+,K^+-ATPase by ouabain *in vitro* (average and 1 SE of 4 experiments).

Table 2 Biodistribution in guinea pigs at 1 hr after i.v. administration*

Compound	% Injected dose / g of tissue					Ratio
	Blood	Heart	Lung	Liver	Kidney	Heart / Blood
^{67}Ga-DFO-digoxin conjugate	0.449 ± 0.073	0.474 ± 0.062	0.411 ± 0.062	1.512 ± 0.294	4.880 ± 1.648	1.076 ± 0.086
^{67}Ga-DFO	0.507 ± 0.050	0.267 ±0.030	0.479 ± 0.057	0.175 ± 0.017	5.321 ± 2.340	0.528 ± 0.030
^{67}Ga-citrate	2.105 ± 0.171	0.830 ± 0.058	1.249 ± 0.107	0.649 ± 0.025	1.822 ± 0.250	0.394 ± 0.042

* Mean ± SE of four animals

DISCUSSION

Because digoxin is too small a molecule to be antigenic by itself, digoxin and its derivatives as haptens were caused to conjugate with a large-molecular carrier. Based on the reaction sequence of Butler and Chen,[9] the carboxy-groups of digoxin dioxime[3] were reacted with the amino-group of deferoxamine, using the activated ester method.

^{1}H NMR spectra and the line graph by continuous variation method indicated that the chelating ability of two DFO sites bound in the DFO-digoxin conjugate was preserved. The results of the binding-test to the anti-digoxin antibody suggested that DFO-digoxin conjugate maintained binding affinity to the antibody.

Since the sensitivity of RIA is dependent on precise measurement of the binding as radioactivity, the improvement in the specific activity of radiolabeled antigen increases the assay sensitivity. ^{67}Ga has a short half life, and considering the theoretical half life alone an 18 fold increase is possible (^{67}Ga: 3.3 vs. ^{125}I: 60 days). In our study, the ^{67}Ga labeled compound had a higher specific activity (159 MBq/nmol) than the ^{125}I labeled compound from the commercially available RIA kit (80 MBq/nmol). Further improvement in the specific activity could be achieved if a generator producing ^{68}Ga with a half life of 68.3 minutes were available. Moreover, the short half-life of radiogallium (^{67}Ga, ^{68}Ga) contributes to the reduction of radioactive waste disposal problems.

The standard cruve of ^{67}Ga-RIA was obtained with the synthesized ^{67}Ga-DFO-digoxin conjugate, and this suggested that ^{67}Ga-DFO-digoxin conjugate retained immunoreactivity to anti-digoxin antibody.

In guinea-pig kidney Na^+,K^+-ATPase, ouabain displacement studies suggested that ^{67}Ga-DFO-digoxin conjugate retained its binding ability to Na^+,K^+-ATPase in spite of the chelation of ^{67}Ga at the carrier level with DFO.

Previously, Misra et al.[10] synthesized three ^{99m}Tc labeled cardiac glycoside derivatives with cymarin, convallotoxin, and strophanthidin-D-glucoside to develop a potential myocardial imaging agent. In their work, thiosemicarbazone as the metal binding site for ^{99m}Tc labeling was attached to an aldehyde group placed in the C-19 position of the steroid aglycone in cardiac glycosides. Their biodistribution studies of three ^{99m}Tc-labeled compounds in guinea pigs showed a lower heart/non-target (except kidney) ratio than that of ^{67}Ga-DFO-digoxin conjugate. We suspected that the metal binding site had little chelating ability with ^{99m}Tc and the modification of the steroid ring for radiometallic labeling would be a big problem in preserving the binding affinity.

In conclusion, ^{67}Ga-DFO-digoxin conjugate retained enough affinity with and specificity for anti-digoxin antibody as well as Na^+,K^+-ATPase, even thought deferoxamine (M.W.=560.71) is a comparatively large molecule (digoxin: M.W.=708.95). The ^{67}Ga-DFO-digoxin conjugate with high specific activity was applicable to digoxin RIA, and the ^{67}Ga-RIA for routine practice may provide a solution to some of the problems inherent in ^{125}I-RIA. ^{67}Ga-DFO-digoxin conjugate would be also a candidate for *in-vivo* Na^+,K^+-ATPase imaging agent. In addition, our studies are applicable for the ^{67}Ga labeling method at high specific activity in other compounds with sugar residues.

REFERENCES

1. Fujibayashi Y, Takemura Y, Matumoto K, et al: High myocardial accumulation of radioiodinated digoxin derivative: a possible Na,K-ATPase imaging agent. *J Nucl Med* 33: 545–549, 1992
2. Yokoyama A, Yoshiro O, Horiuchi K, et al: Deferoxamine, a promising bifunctional chelating agent for labeling proteins with gallium: Ga-67-DF-HSA. *J Nucl Med* 23: 909–914, 1982
3. Japanese patent: B2. S58–51626: 133–140
4. Hwang DR, Scott ME, Hedaya E: Synthesis and characterization of digoxin-phospholipid conjugates. *Bioconjugate Chem* 1: 309–313, 1990
5. Martell E, Calvin M: Methods of determining the presence of chelates. In *Chemistry of the metal chelate compounds*. New York, Prentice-Hall, Inc., pp 28–31, 1953
6. Furukawa T, Fujibayashi Y, Fukunaga M, et al: Ga-labeling of IgG with high specific radioactivity. *Chem Pharm Bull* 38: 2285–2286, 1990
7. Matui H, Schwartz A: Mechanism of cardiac glycoside inhibition of the (Na^+,K^+)-ATPase from cardiac tissue. *Biochim Biophys Acta* 151: 655–663, 1968
8. Post RL, Sen AK: Sodium and potassium-stimulated ATPase. In *Meth Enzymol* 10: pp 762–768, 1967
9. Butler VP, Chen JP: Digoxin-specific antibodies. *Proc Natl Acad Sci* 57: 71–78, 1967
10. Misra M, Sarkar HS, Chatterjee M, et al: Preparation and evaluation of technetium-99m labeled cardiac glycoside derivatives as potential myocardial imaging agent. *Nucl Med Biol* 15 (4): 409–417, 1988