

## Development of Radioimmunoassay for Measurement of Serum Digoxin in Digitalized Patients Using Novel Anti-digoxin Antiserum

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There is an antiserum elicited by digoxin 3'-hemisuccinate–bovine serum albumin (BSA) conjugate possessing high specificity for digoxin. Our study focused on development of RIA using this novel antiserum for measurement of digoxin in serum from digitalized patients. The property of the new antiserum was investigated by RIA with digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine. The separation of bound and free fractions was performed using a dextran-coated charcoal suspension. The new antiserum bound approximately 50% of digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine with a final dilution of 1 : 30000. The intra- and inter-assay coefficients of variation were <9% in the range of 0.52–4.17 ng/ml. The mean digoxin concentration in serum samples (*n*=35) from digitalized patients was estimated to be 0.68 ng/ml, which was lower than its measurement of digoxin with the commercial anti-digoxin BSA serum and monoclonal anti-digoxin. It is apparent that the RIA described here has sufficient precision. The RIA system was available for the measurement of digoxin in serum from digitalized patients.

**Key words** digoxin; anti-digoxin antiserum; RIA; digoxin 3'-hemisuccinate–bovine serum albumin (BSA) conjugate

Digoxin is the most widely used cardiac glycoside for the treatment of congestive heart failure and atrial fibrillation. Digoxin is known to be converted metabolically to digoxigenin bis-digitoxoside, digoxigenin mono-digitoxoside and digoxigenin by cleavage of the sugar moiety.<sup>1,2)</sup> In addition, it is metabolized to dihydrodigoxin by reduction of the double bond in the lactone ring.<sup>3)</sup>

The analysis of digoxin in biological fluids can be performed by immunoassays. The antisera used for these immunoassays are produced by immunization with a hapten-carrier protein conjugate, which is coupled to the terminal digitoxose of digoxin using periodate oxidation.<sup>4,5)</sup> These antisera show high cross-reactivity with metabolites formed by the successive cleavage of the digitoxose residues.<sup>6–8)</sup>

In the radioimmunoassay (RIA) for determining digoxin concentrations in serum, <sup>3</sup>H- and <sup>125</sup>I-labeled digoxin derivatives have been widely used.<sup>8–10)</sup> We recently reported the preparation and antigenic properties of digoxin–bovine serum albumin (BSA) conjugates linked at the digitoxose C-3' and C-3'' positions.<sup>11,12)</sup> The properties of the antisera were characterized by RIA for [<sup>3</sup>H]-digoxin. Although the antiserum elicited by digoxin 3'-hemisuccinate–BSA conjugate possesses high specificity for digoxin, it still lacks the sensitivity needed for measurement of digoxin concentrations in serum. The sensitivity of RIA increases using a tracer whose specific radioactivity is high. In the present study, we describe development of a type of RIA of digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine using the antiserum elicited by digoxin 3'-hemisuccinate–BSA conjugate for measuring digoxin in serum from digitalized patients, and compare its performance with commercially available anti-digoxin BSA serum and monoclonal anti-digoxin.

### MATERIALS AND METHODS

**Materials** L-[3,4,5-<sup>3</sup>H(N)]-Leucine (5328.0 GBq/mmol) was supplied by New England Nuclear (Boston, MA, U.S.A.). Dihydrodigoxin was obtained from Boehringer Mannheim (Mannheim, Germany), Atomlight from Packard

BioScience B. V. (Groningen, The Netherlands), and BSA (fraction V) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Digoxin was purchased from Aldrich (Milwaukee, WI, U.S.A.), Amberlite XAD-2 resin from Rohm and Haas Co. (Philadelphia, PA, U.S.A.), and Dextran T-70 from Pharmacia Fine Chemicals (Uppsala, Sweden). Norit SX and other general reagents were supplied by Wako Pure Chemical Industries (Osaka, Japan). Digoxigenin, and its mono- and bis-digitoxosides were prepared by hydrolysis of digoxin according to the methods of Kaiser and his colleagues.<sup>13)</sup>

RIA was performed in phosphate saline buffer (pH 7.4) containing K<sub>2</sub>HPO<sub>4</sub> (0.696 g), NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O (0.138 g), NaCl (4.39 g), and BSA (1.0 g) in H<sub>2</sub>O (500 ml). A dextran-coated charcoal suspension was prepared by continuously stirring Norit SX (500 mg) and Dextran T-70 (50 mg) in cold phosphate saline buffer (40 ml) for 10 min prior to use.

The antiserum (antibody (A)) was prepared by immunizing rabbit with digoxin 3'-hemisuccinate–BSA conjugate.<sup>12)</sup> The commercial anti-digoxin BSA serum (antibody (B)) and monoclonal anti-digoxin (antibody (C)) were supplied by BioMakor Co. (Rehovot, Israel).

**Apparatus** The melting point was determined with a Yanagimoto micro hot-stage apparatus and is uncorrected. Optical rotation was measured with a JASCO DIP-370 digital polarimeter. FAB-MS measurement was made on a JEOL HX-100 instrument equipped with a FAB ion source using glycerol and NaCl as the matrix agents. The UV spectrum was obtained with a Shimadzu UV-3000 recording spectrophotometer. <sup>1</sup>H-NMR spectra were recorded using tetramethylsilane as an internal standard for a JEOL GSX-400 spectrometer at 400 MHz. Abbreviations used: s=singlet, d=doublet, and m=multiplet.

**Digoxin 3'-Hemisuccinylleucine (4)** Digoxin 3'-hemisuccinate *p*-nitrophenyl ester (**3**) was prepared from digoxin 3'-hemisuccinate (**2**).<sup>12)</sup> A solution of L-leucine (55 mg, 0.42 mmol) in H<sub>2</sub>O (10 ml) was added to a solution of **3** (115 mg, 0.11 mmol) in pyridine (10 ml), and the mixture was stirred at room temperature for 18 h. The solution was extracted with AcOEt, and the aqueous layer was percolated

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through an Amberlite XAD-2 column (47×1.5 cm i.d.). The column was washed with H<sub>2</sub>O, then the desired compound was eluted with MeOH. After evaporation of the MeOH fraction, the crude product obtained was submitted to silica-gel column (37×1.0 cm i.d.) chromatography using CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (80:20:2.5, v/v/v) as a mobile phase and further purified on a Sephadex LH-20 column (81×1.5 cm i.d.) using MeOH as an eluent. The eluate was recrystallized from acetone-hexane to give **4** (37 mg, 34%) as a colorless amorphous solid. mp 175–178 °C.  $[\alpha]_D^{23} +33.0^\circ$  (*c*=0.21, MeOH). *Anal.* Calcd for C<sub>51</sub>H<sub>79</sub>NO<sub>18</sub>·2H<sub>2</sub>O: C, 59.46; H, 8.12; N, 1.36. Found: C, 59.48; H, 7.96; N, 1.38. FAB-MS *m/z*: 1016 [M+Na]<sup>+</sup>. UV  $\lambda_{\max}$  (MeOH) nm ( $\epsilon$ ): 215 (16000). <sup>1</sup>H-NMR (CD<sub>3</sub>OD)  $\delta$ : 0.78 (3H, s, 18-CH<sub>3</sub>), 0.90–0.99 (9H, m, 19- and leucine-CH<sub>3</sub>), 1.13–1.26 (9H, unresolved d, digitoxose-CH<sub>3</sub>), 2.61 (4H, s, -CO(CH<sub>2</sub>)<sub>2</sub>CO-), 4.94 (2H, m, 21-CH<sub>2</sub>), 5.32 (1H, m, 3'-H), 5.91 (1H, s, 22-H).

**Digoxin 3'-Hemisuccinyl-[<sup>3</sup>H]Leucine (5)** A solution of [<sup>3</sup>H]leucine (1.0  $\mu$ Ci) in EtOH-H<sub>2</sub>O (2:98, v/v) (1.0 ml) was added to a solution of **3** (7.0 mg) in pyridine (1.0 ml), and the mixture was stirred at room temperature for 19 h. After addition of H<sub>2</sub>O (18 ml), the reaction mixture was passed through the Sep-Pak C<sub>18</sub> cartridge (weight of packed material, 360 mg/cartridge). After washing with 30% MeOH (50 ml), MeOH (5 ml) was passed through the cartridge. The MeOH fraction was evaporated and dissolved in CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (90:10:0.8, v/v/v) (2 ml), and loaded on the Sep-Pak silica cartridge (weight of packed material, 690 mg/cartridge). After washing with CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (90:10:0.8, v/v/v) (48 ml), **5** was eluted with CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (85:15:1, v/v/v) (20 ml). This clean-up procedure with a Sep-Pak silica cartridge was repeated 3 times and the resulting labeled compound was stored in MeOH at -18 °C.

**RIA Procedure** For the digoxin free serum sample pool serum from healthy volunteers who had not taken any drug was used. The digoxin free serum (0.2 ml), digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine (*ca.* 10000 dpm) diluted with the assay buffer (0.1 ml), and diluted antiserum (0.3 ml) were added to tubes containing from 0 to 15.65 ng/ml of non-labeled digoxin in assay buffer (0.1 ml). All tubes were shaken in a vortex mixer and incubated at 4 °C for 3 h. A dextran-coated charcoal suspension (0.3 ml) was added to each tube, which was then vortexed, incubated for 10 min in an ice bath and centrifuged at 1700×*g* for 10 min at 4 °C. The supernatant (0.7 ml) was transferred to a counting vial, scintillation solution (Atomlight, 6 ml) was added, and the radioactivity was counted in an Aloka LSC-3000 liquid scintillation counter. The radioactivity of the bound antibody was calculated after correction for the blank value (assay buffer). The dose-response curve was constructed using duplicate samples.

**Intra- and Inter-Assay Precision** The intra- and inter-assay precision using antibody (A) was assessed using the digoxin free serum samples to which were added from 0.52 to 4.17 ng/ml of digoxin.

**Cross-Reaction Study** The specificities of antibodies (A), (B), and (C) were tested by calculating the percentage of cross-reactivity with various compounds.<sup>14)</sup> Cross-reactivity was determined by the abovementioned assay procedure, by comparing the concentrations of non-labeled digoxin and test

compounds necessary for a 50% displacement of the antibody-bound labeled digoxin.

**Interference of Digoxin Metabolites with the Assay** A mixture of digoxin and its metabolites was prepared as follows: digoxigenin, its mono- and bis-digitoxoside each with digoxin concentration of 10% were added to digoxin solutions of 0.5, 1, and 2 ng/ml. This mixture was added to the digoxin free serum samples and digoxin concentration was analyzed by RIA using antibody (A), (B), or (C).

**Determination of Digoxin in Serum from Digitalized Patients** The patient serum samples were obtained during routine monitoring of serum digoxin concentrations or other laboratory tests of digitalized patients in Saiseikai Kanazawa Hospital. The samples were stored at -18 °C until assayed. Patient serum sample (0.2 ml), assay buffer (0.1 ml), digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine (0.1 ml), and diluted antiserum (0.3 ml) were added to test tubes. Digoxin concentration in the sample of 0.2 ml was determined by RIA as described above.

## RESULTS AND DISCUSSION

The radiochemical purity of digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine was checked by TLC. TLC separation and the radio-chromatogram are shown in Fig. 1. Digoxin 3'-hemisuccinylleucine (**4**) used as a standard was prepared from digoxin 3'-hemisuccinate *p*-nitrophenyl ester (**3**), which was derived from **2** (Chart 1). The *R<sub>f</sub>* values of L-leucine, digoxin 3'-hemisuccinylleucine, and digoxin 3'-hemisuccinate were 0.18, 0.35, and 0.47, respectively. Also, the radioactivity of digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine correlated well with the *R<sub>f</sub>* value of digoxin 3'-hemisuccinylleucine.

The properties of antibodies (A), (B), and (C) were investigated by RIA with digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine. The separation of bound and free fractions was performed using a dextran-coated charcoal suspension. Antibodies (A), (B), and (C) bound approximately 50% of digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine with a final dilution of 1:30000, 1:46000, and 1:160000, respectively. The association constants of these antibodies were determined to be 1.8×10<sup>9</sup>, 2.7×10<sup>10</sup>, and 8.4×10<sup>10</sup> M<sup>-1</sup> from a Scatchard plot.<sup>15)</sup> The standard curves obtained with antibodies (A), (B), and (C) are presented in Fig. 2. The plot of percent bound radioactivity vs. logarithm of the amount of non-labeled digoxin showed a linear relationship over the range of 0.03 to 1.5 ng

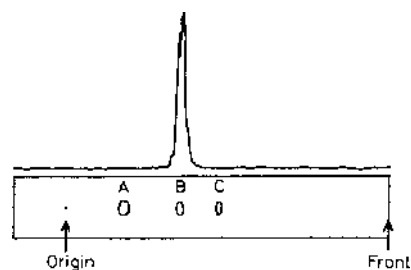


Fig. 1. Thin-Layer Chromatogram (Below) and Radio-Chromatogram (Above) of Labeled Digoxin

TLC specifications: plate, Merck silica-gel 60 F<sub>254</sub>; developing solvent, CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (75:25:3.5, v/v/v). Visualization: spraying with ninhydrin and concentrated sulfuric acid followed by heating in an oven at 120 °C for 10 min. A, L-leucine; B, digoxin 3'-hemisuccinylleucine; and digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine; C, digoxin 3'-hemisuccinate.

Table 1. Precision of the RIA System Using Antibody (A)

Added (ng/ml)	Intra-assay ( $n=8$ )				Inter-assay ( $n=8$ )			
	Mean (ng/ml)	S.D. (ng/ml)	CV (%)	Recovery (%)	Mean (ng/ml)	S.D. (ng/ml)	CV (%)	Recovery (%)
0.52	0.48	0.03	6.3	92.3	0.60	0.05	8.3	115.4
1.05	1.08	0.06	5.6	102.9	1.10	0.09	8.2	104.8
2.09	2.21	0.08	3.6	105.7	2.16	0.16	7.4	103.3
4.17	4.29	0.12	2.8	102.9	4.23	0.30	7.1	101.4

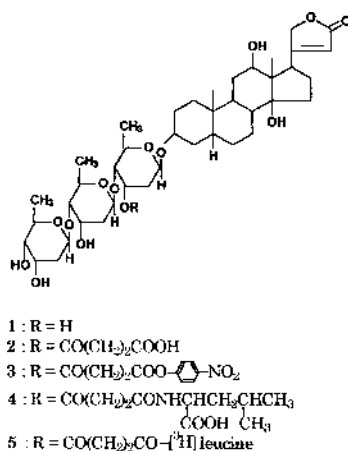


Chart 1

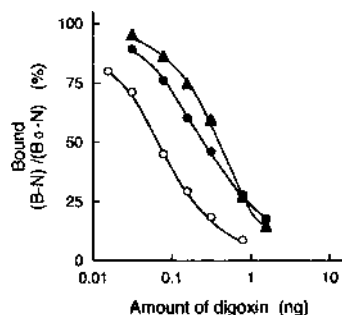


Fig. 2. Standard Curves for Digoxin Using Anti-digoxin Antibodies Antibodies (A) (●), (B) (○), and (C) (▲).

for antibodies (A) and (C), while the plot was linear over the range of 0.02 to 0.7 ng for antibody (B).

The precision of RIA using antibody (A) was evaluated. The intra-, inter-assay, and recovery are presented in Table 1. The intra-assay coefficients of variation (CV) were 2.8–6.3% ( $n=8$ ); the inter-assay CV was 7.1–8.3% ( $n=8$ ). The recovery of various amounts of digoxin added to the digoxin free serum was satisfactory.

The specificities of antibodies (A), (B), and (C) were assessed by cross-reaction tests with various related compounds. Percentages indicating cross-reactivities at 50% displacement of the antibody-bound digoxin 3'-hemisuccinyl-[<sup>3</sup>H]leucine compared to digoxin were calculated and the results are listed in Table 2. Antibody (A) possessed high specificity, exhibiting minor cross-reactions with dihydrodigoxin (9.4%), and digitoxin (6.1%). Also, there were no significant cross-reactions with digoxigenin bisdigitoxoside (0.34%), digoxigenin monodigitoxoside (0.11%), or digoxigenin (0.02%). All other compounds tested showed negli-

Table 2. Cross-Reaction Data of Anti-digoxin Antibody

Compound	% Cross-reactivity (50%) <sup>a)</sup>		
	Antibody (A)	Antibody (B)	Antibody (C)
Digoxin	100	100	100
Digoxigenin bisdigitoxoside	0.34	111	91.9
Digoxigenin monodigitoxoside	0.11	122	120
Digoxigenin	0.02	82.2	106
Dihydrodigoxin	9.4	0.8	4.3
Digitoxin	6.1	0.6	8.7
Spironolactone	<0.01	<0.01	<0.01
Digitoxose	<0.01	<0.01	<0.01
Progesterone	<0.01	<0.01	<0.01
Testosterone	<0.01	<0.01	<0.01
Cholesterol	<0.001	<0.001	<0.001

a) Values are calculated on a molar basis.

ble values of <0.01%. In contrast, antibodies (B) and (C) showed high cross-reactivity to digoxin degradation products. Antibody (A) is highly specific for both the digitoxose chain and the steroid portion of the molecule. However, antibodies (B) and (C) were inferior in terms of specificity with respect to changes in the digitoxose part or the steroid moiety of digoxin.

A mixture of digoxin, digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside and digoxigenin was added to the digoxin free serum and digoxin concentration was analyzed by RIA using antibody (A), (B), or (C). Recoveries for digoxin by antibodies (A), (B), and (C) were 100.0–101.5%, 122.0–144.0%, and 121.5–154.0%, respectively (Table 3). Antibody (A) recovers digoxin closest to the original analyte spike level, but antibodies (B) and (C) recover much higher digoxin than the digoxin spike level; this may be due to the high cross-reactivity of these two antibodies with the digoxin metabolites present in the mixtures.

The serum samples ( $n=35$ ) obtained from digitalized patients were measured by RIA using antibody (A) and its performance was compared with RIAs using antibodies (B) and (C), respectively (Fig. 3). When antibody (A) was used, the mean digoxin concentration was 0.68 ng/ml (range, 0.24–1.50 ng/ml). Then, the same samples were measured with antibodies (B) and (C), and the mean values of digoxin were 0.79 ng/ml (range, 0.34–1.79 ng/ml) and 0.80 ng/ml (range, 0.32–1.85 ng/ml), respectively. We suggest that the digoxin measurement results detailed with antibody (A) are accurate. On the other hand, the values obtained using antibodies (B) and (C) were higher than that of antibody (A). It is possible that the RIA using antibodies (B) and (C) measures not only digoxin but also its metabolites. We surmised from the dis-

Table 3. Interference of Digoxin Metabolites on the Assay

Added	Antibody (A)		Antibody (B)		Antibody (C)	
	Mean±S.D. (ng/ml) (n=5)	Recovery (%)	Mean±S.D. (ng/ml) (n=5)	Recovery (%)	Mean±S.D. (ng/ml) (n=5)	Recovery (%)
Digoxin (0.5 ng/ml)+ each metabolite (0.05 ng/ml)	0.50±0.10	100.0	0.72±0.03	144.0	0.77±0.05	154.0
Digoxin (1 ng/ml)+ each metabolite (0.1 ng/ml)	1.00±0.06	100.0	1.22±0.05	122.0	1.23±0.03	123.0
Digoxin (2 ng/ml)+ each metabolite (0.2 ng/ml)	2.03±0.06	101.5	2.47±0.10	123.5	2.43±0.08	121.5

Metabolites are digoxigenin, digoxigenin monodigitoxoside, and digoxigenin bisdigitoxoside.

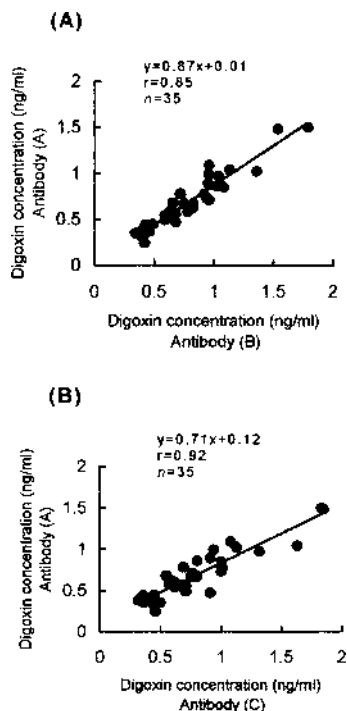


Fig. 3. Plots of Values Assaying Serum Samples of Digitalized Patients (A) A comparison of antibody (A) and (B). (B) A comparison of antibody (A) and (C).

persion of measured value that individual differences between patients exist with regard to rate of metabolism from digoxin to digoxigenin, its mono- and bis-digitoxoside, and dihydrodigoxin.

Immunoassays are typically used for therapeutic drug monitoring of the cardiac glycosides, and in these immunoassays, antibody with cross-reactivity to metabolites of

this drug matching the drug's biological activities should be used.<sup>7,8,16-18</sup> It should be mentioned that the RIA developed here does not meet this requirement. However, this RIA using antibody (A) measures unmetabolized digoxin, and may be utilized for pharmacokinetics studies. Antibody (A) may also be useful for therapeutic immunoextraction of digoxin in patients with digoxin overdoses.

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