# Preparation of Specific Antisera to Digoxin by Using Digoxin C-3′ and C-3″ Hemisuccinate–Bovine Serum Albumin Conjugates

Yukari IKEDA* and Youichi FUJII

*Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3, Kanagawa-machi, Kanazawa 920–1181, Japan.*
Received February 21, 2000; accepted May 8, 2000

**The preparation and antigenic properties of digoxin C-3′ and C-3″ hemisuccinate–bovine serum albumin (BSA) conjugates are described. The antisera were prepared by immunizing rabbits with each of the digoxin–BSA conjugates, and properties of the antisera were characterized by RIA with $^3$H-labeled digoxin. The anti-digoxin antiserum from immunization with digoxin 3′-hemisuccinate–BSA conjugate possessed high specificity for digoxin, exhibiting fairly low cross-reactions with dihydrodigoxin (2.1%), digoxigenin monodigitoxoside (0.9%), digoxigenin bisdigitoxoside (0.6%), and digoxigenin (0.1%).**

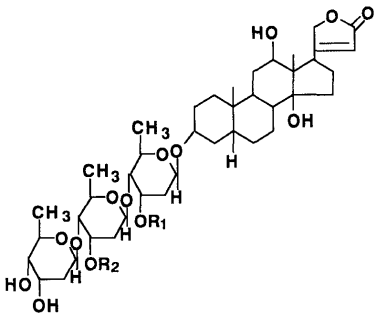
**Key words** digoxin; anti-digoxin antiserum; digoxin–BSA conjugate; RIA; digoxin hemisuccinate

Digoxin, the most commonly used digitalis glycoside, is an important drug for the treatment of congestive heart failure and atrial fibrillation. Several immunoassay procedures have been used to monitor serum digoxin concentrations in the toxic or therapeutic range. The antisera used in immunoassay were produced by immunization with a hapten–carrier protein conjugate that was coupled through the terminal digitoxose of digoxin by periodate oxidation.[1,2] The antisera showed high cross-reactivities with metabolites formed by the successive cleavage of the digitoxose residues (*i.e.* digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, and digoxigenin).[3—5] For the purpose of obtaining specific antisera that can distinguish between digoxin and its metabolites, Shimada *et al.*[6] and Thong *et al.*[7] used conjugates where the hapten was linked to the carrier protein at the C-12, C-17, and C-22 positions, respectively. However, these antisera exhibited remarkable cross-reactivity with dihydrodigoxin, one of the metabolites.

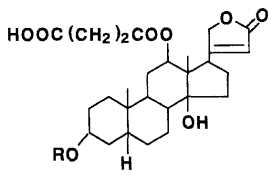
The specificity of antisera is strongly influenced by the position on the cardiac glycoside molecule linked to an immunogenic carrier protein. Previously, we reported preparation and antigenic properties of digitoxin–bovine serum albumin (BSA) conjugates linked at the digitoxose C-3′ and C-3″ positions.[8,9] The requirement for much more specific antisera for the immunoassay of digoxin prompted us to develop new haptens. We present here the preparation of specific anti-digoxin antisera that can discriminate digoxin from its metabolites. The rabbit antisera were elicited with antigens which were synthesized digoxin hemisuccinate–BSA conjugates possessing bridges at the C-3′ and C-3″ positions in the digitoxose chain, remote from both the terminal digitoxose and the steroid nucleus. The characterization of the antisera elicited by these antigens was performed by RIA with $^3$H-labeled digoxin. This paper comprises the full details of the preparation of antiserum using digoxin 3′-hemisuccinate–BSA conjugate.[10]

## MATERIALS AND METHODS

**Materials** [$^3$H(G)]Digoxin (836.2 GBq/mmol) and scintillation solution (Atomlight) were supplied by New England Nuclear (Boston, MA, U.S.A.). Dihydrodigoxin and dihydrodigitoxin were obtained from Boehringer Mannheim (Mannheim, Germany), spironolactone and BSA (fraction V) from Sigma Chemical Co. (St. Louis, MO, U.S.A.), digoxin from Aldrich (Milwaukee, WI, U.S.A.), and Freund's complete adjuvant from Difco Lab. (Detroit, MI, U.S.A.). Sephadex LH-20 and dextran T-70 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), reversed-phase $KC_{18}$ plates (5×10 cm) for TLC from Whatman (Clifton, NJ, U.S.A.), Kiesel-gel 60 for column chromatography and high performance thin-layer chromatography (HPTLC) plates (5×10 cm) from E. Merck (Darmstadt, Germany). Digitoxin 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



**1** : $R_1 = R_2 = H$
**2** : $R_1 = CO(CH_2)_2COOH$; $R_2 = H$
**3** : $R_1 = H$; $R_2 = CO(CH_2)_2COOH$
**4** : $R_1 = CO(CH_2)_2COO$–⟨⟩–$NO_2$; $R_2 = H$
**5** : $R_1 = H$; $R_2 = CO(CH_2)_2COO$–⟨⟩–$NO_2$
**6** : $R_1 = CO(CH_2)_2CONH$-BSA; $R_2 = H$
**7** : $R_1 = H$; $R_2 = CO(CH_2)_2CONH$-BSA



**8** : R = $(digitoxose)_3$
**9** : R = H

Chart 1

* To whom correspondence should be addressed. e-mail: y-ikeda@hokuriku-u.ac.jp

co-workers.[11]

RIA was performed in phosphate saline buffer (pH 7.4) containing $K_2HPO_4$ (0.696 g), $NaH_2PO_4 \cdot H_2O$ (0.138 g), NaCl (4.39 g), and bovine gamma-globulin (1.0 g) in $H_2O$ (500 ml). A dextran-coated charcoal suspension was prepared by continuously stirring Norit A (250 mg) and dextran T-70 (25 mg) in cold phosphate saline buffer (40 ml) for 10 min prior to use.

**Apparatus** All melting points were determined with a Yanagimoto micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. FAB-MS measurements were made on a JEOL HX-100 instrument equipped with a FAB ion source using glycerol and NaCl as the matrix agents. UV spectra were obtained with a Shimadzu UV-3000 recording spectrophotometer. $^1$H-NMR spectra were recorded using tetramethylsilane as an internal standard on a JEOL GSX-400 spectrometer at 400 MHz. Abbreviations used: s=singlet, d=doublet, and m=multiplet.

**Digoxin 3′-Hemisuccinate (2) and Digoxin 3″-Hemisuccinate (3)** To a solution of digoxin (1.0 g, 1.3 mmol) in pyridine (10 ml), a mixture of formic acid and acetic anhydride (2 : 1, v/v) (6 ml) was added under ice-cooling, and the whole was stirred for 4 h. The reaction mixture was extracted with AcOEt and the extract washed with $H_2O$. After evaporation of the solvent, the residue was dissolved in pyridine (20 ml), succinic anhydride (3 g, 30 mmol) was added, and the mixture was allowed to stand at 70 °C for 73 h. The reaction mixture was extracted with AcOEt, the extract washed with 1% HCl, 1% $NaHCO_3$, and $H_2O$, then dried over anhydrous $Na_2SO_4$. After evaporation of the solvent, the residue was dissolved in MeOH (100 ml), then 5% $NaHCO_3$ (50 ml) was added. The mixture was allowed to stand at 60—70 °C for 3 h, then acidified by adding AcOH, and most of the MeOH was removed under reduced pressure. The resulting solution was extracted with AcOEt, and the organic layer was washed with $H_2O$ and then dried over anhydrous $Na_2SO_4$. After evaporation of the solvent, the crude product obtained was submitted to silica-gel column (84×1.5 cm i.d.) chromatography using $CHCl_3$–MeOH–$H_2O$ (90 : 10 : 0.8, v/v) to give fractions 1 (420—600 ml) and 2 (660—800 ml). Fraction 2 was rechromatographed on a silica-gel column using $CHCl_3$–MeOH–AcOH (90 : 10 : 0.8, v/v) as a mobile phase, and was further purified on a Sephadex LH-20 column (83×1.5 cm i.d.) using MeOH as an eluent. The eluate was recrystallized from acetone–hexane to give **2** (101 mg, 9.2%) as a colorless amorphous solid. mp 175—178 °C. $[\alpha]_D^{31}$ +39.4°(*c* 0.27, MeOH). *Anal.* Calcd for $C_{45}H_{68}O_{17}$: C, 61.35; H, 7.78. Found: C, 61.41; H, 7.48. FAB-MS *m/z*: 903 $[M+Na]^+$, 621 [digoxigenin monodigitoxoside 3′-hemisuccinate+H]$^+$. UV $\lambda_{max}$ (MeOH) nm ($\varepsilon$): 218 (15000). $^1$H-NMR ($CD_3OD$–$CDCl_3$) $\delta$: 0.78 (3H, s, 18-$CH_3$), 0.94 (3H, s, 19-$CH_3$), 1.16—1.25 (9H, unresolved d, digitoxose-$CH_3$), 2.52—2.64 (4H, m, –$CO(CH_2)_2$ CO–), 4.94 (2H, m, 21-$CH_2$), 5.90 (1H, s, 22-H). Fraction 1 was rechromatographed on a silica-gel column (82×1.5 cm i.d.) using $CHCl_3$–MeOH–AcOH (90 : 10 : 0.8, v/v) as a mobile phase and further purified on a Sephadex LH-20 column (83×1.5 cm i.d.) using MeOH as an eluent. The eluate was recrystallized from acetone–hexane to give **3** (189 mg, 16%) as a colorless amorphous solid. mp 176—179 °C. $[\alpha]_D^{31}$ +37.7° (*c* 0.27, MeOH). *Anal.* Calcd for $C_{45}H_{68}O_{17}$: C, 61.35; H, 7.78. Found: C, 61.07; H, 8.01. FAB-MS *m/z*: 903 $[M+Na]^+$. UV $\lambda_{max}$ (MeOH) nm ($\varepsilon$): 218 (14700). $^1$H-NMR ($CD_3OD$–$CDCl_3$) $\delta$: 0.78 (3H, s, 18-$CH_3$), 0.95 (3H, s, 19-$CH_3$), 1.18—1.23 (9H, unresolved d, digitoxose-$CH_3$), 2.56—2.63 (4H, m, –$CO(CH_2)_2$ CO–), 4.94 (2H, m, 21-$CH_2$), 5.90 (1H, s, 22-H).

**Digoxin 3′-Hemisuccinate *p*-Nitrophenyl Ester (4) and Digoxin 3″-Hemisuccinate *p*-Nitrophenyl Ester (5)** Dicyclohexylcarbodiimide (43 mg, 0.21 mmol) was added to a solution of **2** (83 mg, 0.094 mmol) and *p*-nitrophenol (120 mg, 1.1 mmol) in dioxane (10 ml), and the mixture was stirred at room temperature for 4 h. After evaporation of the solvent, the crude product obtained was submitted to preparative TLC using $CHCl_3$–MeOH (93 : 7, v/v) as a developing solvent. Elution of the zone (*Rf* 0.30) corresponding to the product with $CHCl_3$–MeOH (93 : 7, v/v) gave **4** (85 mg, 90%) as a yellow oil. $^1$H-NMR ($CDCl_3$) $\delta$: 0.80 (3H, s, 18-$CH_3$), 0.93 (3H, s, 19-$CH_3$), 1.15—1.28 (9H, unresolved d, digitoxose-$CH_3$), 2.95 (4H, m, –$CO(CH_2)_2CO$–), 4.72—4.93 (5H, m, 21-$CH_2$ and anomeric H), 5.35 (1H, m, 3′-H), 5.93 (1H, br s, 22-H), 7.27, 8.27 (each 2H, d, *J*=9 Hz, aromatic H). Treatment of **3** (67 mg, 0.076 mmol) in the same manner as **4** gave **5** (69 mg, 91%) as a yellow oil. $^1$H-NMR ($CDCl_3$) $\delta$: 0.80 (3H, s, 18-$CH_3$), 0.93 (3H, s, 19-$CH_3$), 1.14—1.26 (9H, unresolved d, digitoxose-$CH_3$), 2.97 (4H, m, –$CO(CH_2)_2CO$–), 4.78—4.93 (5H, m, 21-$CH_2$ and anomeric H), 5.42 (1H, m, 3″-H), 5.94 (1H, br s, 22-H), 7.29, 8.28 (each 2H, d, *J*=9 Hz, aromatic H).

**Digoxin 3′-Hemisuccinate–BSA Conjugate (6) and Digoxin 3″-Hemisuccinate–BSA Conjugate (7)** A solution of BSA (40 mg) in 0.05 M phosphate buffer (pH 7.0, 0.8 ml) was added to a solution of **4** or **5** (30 mg, 0.030 mmol) in pyridine (0.8 ml) and the mixture was stirred at room temperature for 15 h. The resulting solution was dialyzed against a constant flow of cold water (20 l) at 4 °C overnight. Lyophilization of the solution afforded **6** or **7** (49 mg) as a fluffy powder. The molar digoxin–BSA ratios of **6** and **7** were spectrophotometrically (388 nm) determined from the absorption developed with 92% $H_2SO_4$,[12] and were found to be 36 and 33, respectively.

**Digoxin 12-Hemisuccinate (8)** Succinic anhydride (4.0 g, 40 mmol) was added to a solution of digoxin (2.0 g, 2.6 mmol) in pyridine (40 ml), and the mixture was allowed to stand at 60 °C for 20 h. The reaction mixture was extracted with AcOEt and the extract was washed with 1% HCl, 1% $NaHCO_3$, and $H_2O$, then dried over anhydrous $Na_2SO_4$. After evaporation of the solvent, the residue was redissolved in MeOH (100 ml), then 5% $NaHCO_3$ (100 ml) was added. The mixture was allowed to stand at 60 °C for 2.5 h, then acidified by adding AcOH, and most of the MeOH was removed under reduced pressure. The resulting solution was extracted with AcOEt, and the organic layer was washed with $H_2O$ and then dried over anhydrous $Na_2SO_4$. After evaporation of the solvent, the crude product obtained was submitted to silica-gel column (130×1.7 cm i.d.) chromatography using $CHCl_3$–MeOH–AcOH (92 : 8 : 0.6, v/v) as a mobile phase. The eluate was recrystallized from acetone–hexane to give **8** (299 mg, 13%) as a colorless amorphous solid. mp 168—171 °C. $[\alpha]_D^{21}$ +36.6° (*c* 0.43, MeOH). *Anal.* Calcd for $C_{45}H_{63}O_{17} \cdot H_2O$: C, 60.12; H, 7.85. Found: C, 60.46; H, 8.00. FAB-MS *m/z*: 925 $[M-H+2Na]^+$, 903 $[M+Na]^+$, 773 [digoxigenin bisdigitox-

oside 12-hemisuccinate+H+Na]$^+$. UV $\lambda_{max}$ (MeOH) nm ($\varepsilon$): 218 (13600). $^1$H-NMR ($CD_3OD$) $\delta$: 0.91 (3H, s, 18-$CH_3$), 0.95 (3H, s, 19-$CH_3$), 1.18—1.27 (9H, unresolved d, digitoxose-$CH_3$), 2.63 (4H, m, –$CO(CH_2)_2CO$–), 4.65 (1H, br s, 12$\alpha$-H), 4.97 (2H, m, 21-$CH_2$), 6.00 (1H, s, 22-H).

**Digoxigenin 12-Hemisuccinate (9)** To a solution of **8** (100 mg, 0.11 mmol) in MeOH (10 ml), 0.05 M $H_2SO_4$ (13 ml) was added and the mixture was allowed to stand at 70 °C for 3 h. The reaction mixture was extracted with AcOEt, and the organic layer was washed with $H_2O$ and then dried over anhydrous $Na_2SO_4$. After evaporation of the solvent, the crude product obtained was submitted to Sephadex LH-20 column (80×1.5 cm i.d.) chromatography using hexane–$CHCl_3$–MeOH (5 : 5 : 1, v/v) as a mobile phase. The eluate was rechromatographed on a silica-gel column (45× 0.6 cm i.d.) using $CHCl_3$–MeOH–AcOH (95 : 5 : 0.2, v/v) as a mobile phase and further purified on a Sephadex LH-20 column (80×1.2 cm i.d.) using MeOH as an eluant. The eluate was recrystallized from $CH_2Cl_2$ to give **9** (38 mg, 70%) as a colorless amorphous solid. mp 135—138 °C. $[\alpha]_D^{26}$ +66.5°(*c* 0.32, MeOH). *Anal.* Calcd for $C_{27}H_{38}O_8 \cdot H_2O$: C, 63.76; H, 7.93. Found: C, 63.77; H, 7.93. EI-MS *m/z*: 490 $[M]^+$, 472 $[M-H_2O]^+$, 390 [digoxigenin]$^+$. UV $\lambda_{max}$ (MeOH) nm ($\varepsilon$): 215 (12400). $^1$H-NMR ($CD_3OD$) $\delta$: 0.92 (3H, s, 18-$CH_3$), 0.98 (3H, s, 19-$CH_3$), 2.68 (4H, m, –$CO(CH_2)_2$ CO–), 5.02 (2H, m, 21-$CH_2$), 6.04 (1H, br s, 22-H).

**Partial Hydrolysis of 1, 2, or 3** To a solution of **1**, **2**, or **3** (2 mg) in MeOH (0.52 ml), 0.05 M HCl (0.08 ml) was added, and the mixture was allowed to stand at 60 °C for 1 h and spotted on TLC plates.

**Immunization Procedure** Three domestic strain male albino rabbits were used for immunization with each hapten–BSA conjugate. The antigen (3 mg) was dissolved in sterile isotonic saline (0.9 ml) and emulsified with complete Freund's adjuvant (2.1 ml). The emulsion was injected into multiple subcutaneous sites along both sides of the back of each rabbit. The procedure was repeated at intervals of 2 weeks for 4 months. After confirmation of the increase in the antiboby titer, blood was collected from the marginal ear veins. Sera were separated by centrifugation at 1400×***g*** for 10 min and stored at −18 °C in small aliquots. The antisera were thawed, diluted with phosphate saline buffer (pH 7.4), and used in the assay.

**RIA Procedure** [$^3$H]Digoxin (*ca.* 16000 dpm) in assay buffer (0.1 ml) and diluted antiserum (0.5 ml) were added to test tubes containing 0, 0.01, 0.02, 0.04, 0.1, 0.2, 0.4, 1, 2, 4, and 10 ng of non-labeled digoxin in assay buffer (0.1 ml). All tubes were shaken in a vortex mixer and incubated at 4 °C overnight. A dextran-coated charcoal suspension (0.3 ml) was added to each tube, which was then vortexed, incubated for 10 min in an ice water bath, and centrifuged at 1700×***g*** for 10 min at 4 °C. The supernatant (0.5 ml) was transferred to a counting vial and a scintillation solution (10 ml) added. The tritium radioactivity was measured in a Beckman LS-9000 liquid scintillation spectrometer. The radioactivity bound to the antibody was calculated after correction for the blank value of the assay buffer. The dose-response curve was constructed using duplicate samples.

**Cross-Reaction Study** The specificities of the antisera raised against 2 kinds of digoxin–BSA conjugates were tested by calculating the percentage cross-reactivity with various compounds.[13] Cross-reactivity was determined by the above-mentioned assay procedure, by comparing the concentrations of non-labeled digoxin and test compounds necessary for a 50% displacement of the antibody-bound labeled digoxin.

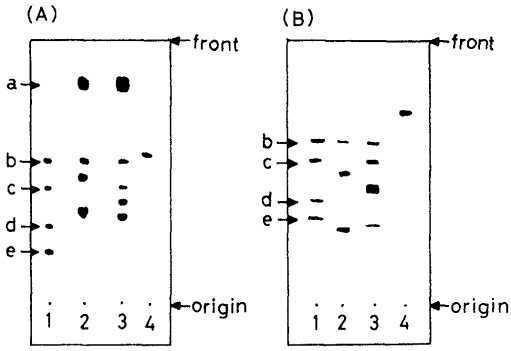



Fig. 1. TLC Chromatogram of Products Obtained by Partial Hydrolysis of Digoxin 3′- and 3″-Hemisuccinates

(A): Whatman $KC_{18}$; developing solvent, MeOH–0.5 M NaCl (18 : 13, v/v). (B): Merck HPTLC (silica-gel); developing solvent, cyclohexane–AcOEt–EtOH–AcOH (15 : 15 : 2 : 2, v/v), development, twice. Visualization: spraying with concentrated sulfuric acid followed by heating in an oven at 120 °C for 10 min. 1, hydrolysate of digoxin; 2, hydrolysate of digoxin 3′-hemisuccinate; 3, hydrolysate of digoxin 3″-hemisuccinate; 4, digoxigenin 12-hemisuccinate. a, hemisuccinates; b, digoxigenin; c, digoxigenin monodigitoxoside; d, digoxigenin bisdigitoxoside; e, digoxin.

## RESULTS AND DISCUSSION

Initially, the synthesis of digoxin 3′- and 3″-hemisuccinate–BSA conjugates (**6** and **7**) was carried out. In the previous paper of this series,[9] we reported the preparation of digitoxin 3′- and 3″-hemisuccinate from digitoxin by utilizing the reactivities of the hydroxyl groups in the digitoxose moiety. In the similar way, digoxin 3′- and 3″-hemisuccinates (**2** and **3**) could be prepared from digoxin (**1**). In acetylation, the reactivity of hydroxyl groups in **1** was in the order C-4‴>C-12>C-3‴>C-3′=C-3″.[14] On the other hand, saponification of the acetyl groups was in the order C-4‴>C-3‴>C-3′=C-3″>C-12.[15] For the preparation of **2** and **3**, protection of the C-12 hydroxyl group in **1** is a prerequisite. After the C-12, C-3‴, and C-4‴ positions of **1** were protected with formyl groups, the mixture of digoxin formates was transformed into their hemisuccinates by succinic anhydride in pyridine. Without purification of the products, the selective saponification of the formyl groups using sodium bicarbonate in aqueous methanol afforded a mixture of **2** and **3** as the main products. The resulting crude product was submitted to silica-gel column chromatography using a chloroform–methanol–water solvent system to furnish a mixture of **2** and **3**. Subsequent column chromatography on silica-gel using chloroform–methanol–acetic acid (90 : 10 : 0.8, v/v) as an eluent provided a satisfactory separation of 2 isomeric hemisuccinates. In the $^1$H-NMR spectra of **2** and **3**, the 4 methylene protons appeared as a multiplet, showing that one succinyl moiety had been introduced. The position of the succinyl group was elucidated by partial hydrolysis of these compounds. TLC of their hydrolyzate is shown in Fig. 1. Digoxigenin 12-hemisuccinate (**9**) used as a standard was prepared from digoxin 12-hemisuccinate (**8**) which was derived from **1** by succinic anhydride in pyridine. When **1** was treated with

0.05 M hydrochloric acid under mild conditions, the glycosidic bond was partially hydrolyzed to give a mixture of digoxigenin, its mono- and bis-digitoxoside, and the intact starting materials. When **2** and **3** were treated in the same way, **9** was not produced from **2** and **3**, digoxigenin only was produced from **2** and both digoxigenin and its monodigitoxoside from **3**. Therefore, **2** and **3** were designated as the 3′-hemisuccinate and 3″-hemisuccinate of digoxin, respectively. The results of other instrumental analyses of these compounds also support these structures.

To couple the hapten with BSA, **2** and **3** were transformed into their *p*-nitrophenyl esters (**4** and **5**) by treatment with *p*-nitrophenol and dicyclohexylcarbodiimide in dioxane. These *p*-nitrophenyl esters were then linked with BSA, followed by dialysis of the reaction mixture against cold water to give the desired BSA conjugates (**6** and **7**). Spectrophotometric analysis revealed that satisfactory numbers of hapten molecules were linked to each BSA molecule in these antigens.

Each immunogen thus obtained was administered to 3 rabbits to produce the antibody. Among the antisera elicited in rabbits, the most specific antiserum to digoxin was selected for characterization in each group. The properties of the antisera were investigated by RIA with tritiated digoxin. The separation of bound and free fractions was performed using a dextran-coated charcoal suspension. The antisera raised against **6** and **7** bound approximately 50% of [$^3$H]digoxin at final dilutions of 1 : 2100 and 1 : 5300, respectively. The association constants of these antisera were determined to be $1.8\times10^9$ and $4.1\times10^8$ M$^{-1}$ from a Scatchard plot.[16] The standard curves obtained with the antisera are presented in Fig. 2. The plots of logit percent bound radioactivity *vs.* logarithm of the amount of non-labeled digoxin showed linear relationships over the range 0.1 to 10 ng.

The specificities of the antisera were assessed by cross-reaction tests with variously related compounds. The percentage cross-reactivities were calculated at 50% displacement of the antibody-bound tritiated digoxin. The antiserum raised against **6** possessed high specificity, exhibiting minor cross-reactions with digitoxin (15.2%), dihydrodigoxin (2.1%), and gitoxin (1.8%). Also, there were no significant cross-reactions with digoxigenin monodigitoxoside (0.9%), digoxigenin bisdigitoxoside (0.6%), and digoxigenin (0.1%). All other compounds tested showed negligible values of <0.05%. In contrast, the antiserum elicited by **7** exhibited considerable cross-reactivity with digoxigenin bisdigitoxoside (85.6%), digoxigenin monodigitoxoside (68.6%), and digoxigenin (60.5%). The results of our antisera and previously reported antisera against digoxin–BSA coupled through the terminal digitoxose (antigen (A)),[5] 12-dehydrodigoxin derivative–BSA at the C-12 position (antigen (B)),[6] and digoxin–BSA *via* the lactone ring (antigen (C))[7] are listed in Table 1. The antiserum against antigen (A) showed high cross-reactivity with metabolites formed by the successive cleavage of the digitoxose residues. Although the antisera against antigens (B) and (C) exhibited minimal cross-reactivities to digoxin degradation products, these antisera showed high cross-reactivity with dihydrodigoxin. It is evident from the cross-reaction data that the antibody produced by an antigen in which the hapten is linked to a carrier protein through the digitoxose C-3′ position, remote from both the terminal digitoxose and the steroid nucleus, is able to recognize both the sugar moiety and the lactone ring of digoxin. However, the antiserum produced by digoxin–BSA conjugate possessing a bridge at the C-3″ position near the terminal digitoxose is inferior in terms of specificity with re-

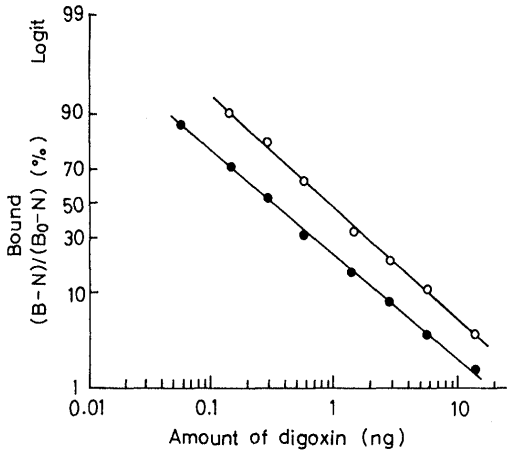



Fig. 2. Standard Curves for Digoxin Using Anti-digoxin Antisera

Digoxin 3′-hemisuccinate–BSA conjugate (●), digoxin 3″-hemisuccinate–BSA conjugate (○).

Table 1. Cross-Reaction Data of Anti-digoxin Antisera

| Compound | % Cross-reactivity (50%)[a] | | | | |
|---|---|---|---|---|---|
| | Digoxin 3′-hemisuccinate–BSA | Digoxin 3″-hemisuccinate–BSA | Antigen (A)[b] | Antigen (B)[b] | Antigen (C)[b] |
| Digoxin | 100 | 100 | 100 | 100 | 100 |
| Digoxigenin bisdigitoxoside | 0.6 | 85.6 | 126 | 0.6 | 14.6 |
| Digoxigenin monodigitoxoside | 0.9 | 68.6 | 123 | 0.2 | 3.7 |
| Digoxigenin | 0.1 | 60.5 | 143 | <0.01 | — |
| Dihydrodigoxin | 2.1 | 0.3 | 1.3 | 65.3 | 67 |
| Digitoxin | 15.2 | 31.3 | — | 29.1 | 11 |
| Gitoxin | 1.8 | 1.0 | — | — | — |
| Spironolactone | <0.05 | <0.05 | — | <0.01 | — |
| Digitoxose | <0.05 | <0.05 | — | — | — |
| Progesterone | <0.05 | <0.05 | — | — | — |
| Testosterone | <0.05 | <0.05 | — | — | — |
| Cholesterol | <0.05 | <0.05 | — | — | — |

*a*) Values are calculated on a molar basis. *b*) Antigen (A): periodate-oxidized digoxin–BSA.[5] Antigen (B): 12-dehydrodigoxin derivative–BSA.[6] Antigen (C): digoxin–BSA *via* the reductive ozonolysis of the lactone ring.[7]

spect to changes in the digitoxose part. In addition, the specificity of the former antiserum to the digitoxose chain investigated by RIA is better than that of the monoclonal antibodies assessed by an immunoenzymatic test.[17]

From the present results and the previous study involving anti-digitoxin antiserum,[8,9] the C-3′ position seems to be a favorable site for the attachment of BSA on the cardiac glycoside in the production of the specific antisera. This antiserum may be used for the determination of intact digoxin in serum from digitalized patients, the immunoaffinity extraction of digoxin, and the therapeutic reversal of digoxin intoxication.

**Acknowledgement** This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

## REFERENCES

1) Butler V. P., Jr., Chen J. P., *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 71—78 (1967).
2) Smith T. W., Butler V. P., Jr., Haber E., *Biochemistry*, **9**, 331—337 (1970).
3) Valdes R., Jr., Brown B. A., Graves S. W., *Am. J. Clin. Pathol.*, **82**, 210—213 (1984).
4) Soldin S. J., *Clin. Chem.*, **32**, 5—12 (1986).
5) Miller J. J., Straub R. W., Jr., Valdes R., Jr., *Clin. Chem.*, **40**, 1898—1903 (1994).
6) Shimada K., Mizusawa S., Ohkubo T., Nambara T., *Chem. Pharm. Bull.*, **32**, 2301—2306 (1984).
7) Thong B., Soldin S. J., Lingwood C. A., *Clin. Chem.*, **31**, 1625—1631 (1985).
8) Fujii Y., Ikeda Y., Yamazaki M., *Clin. Chem.*, **37**, 476—477 (1991).
9) Fujii Y., Ikeda Y., Fujii M., Yamazaki M., *Biol. Pharm. Bull.*, **17**, 467—471 (1994).
10) A preliminary communication: Fujii Y., Ikeda Y., Yamazaki M., *Clin. Chem.*, **40**, 172—173 (1994).
11) Haack E., Kaiser F., Spingler H., *Naturwissenschaften*, **44**, 633—634 (1957).
12) Butler V. P., Jr., Tse-Eng D., Lindenbaum J., Kalman S. M., Preibisz J. J., Rund D. G., Wissel P. S., *J. Pharmacol. Exp. Ther.*, **221**, 123—131 (1982).
13) Abraham G. E., *J. Clin. Endocrinol. Metab.*, **29**, 866—870 (1969).
14) Maerten G., Haberland G., *Arzneim.-Forsch.*, **20**, 347—351 (1970).
15) Schmersahl P., *Arch. Pharm.* (Weinheim)., **305**, 871—875 (1972).
16) Scatchard G., *Ann. N.Y. Acad. Sci.*, **51**, 660—672 (1949).
17) Wahyono D., Piechaczyk M., Mourton C., Bastide J.-M., Pau B., *Hybridoma*, **9**, 619—629 (1990).