

Properties of Novel Anti-digoxin Antisera in Radioimmunoassay Using Homologous and Site Heterologous Tritium-Labeled Antigens Involving a [^3H]-Leucine Moiety

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The specificities of antisera against digoxin C-3' or C-3'' hemisuccinate–bovine serum albumin (BSA) conjugate were assessed by cross-reactivity studies with digoxin metabolites by radioimmunoassay (RIA) using the homologous and the site heterologous tritium-labeled antigens. One of the tracers used was digoxin 3'-hemisuccinyl- ^3H -leucine; the other was digoxin 3''-hemisuccinyl- ^3H -leucine, which had been prepared from digoxin 3''-hemisuccinate. When the tracer with ^3H -leucine at the C-3' position was used, antisera (I-1, I-3) elicited by digoxin 3'-hemisuccinate–BSA conjugate showed the following cross-reactivity: digoxigenin bisdigitoxoside (0.34%, 76%), digoxigenin monodigitoxoside (0.11%, 65%), digoxigenin (0.02%, 26%) and dihydrodigoxin (9.4%, 1.2%). However, when using the homologous antigen, antiserum (I-1) was highly specific against the digitoxose chain. When the site heterologous antigen, digoxin 3''-hemisuccinyl- ^3H -leucine was combined, this antiserum showed high cross-reactivity to digoxin degradation products. This digoxin RIA using antiserum (I-1) with the homologous antigen measures unmetabolized digoxin. On the other hand, the RIA system using antiserum (I-3) with the homologous antigen had cross-reactivity with the metabolites in accordance with their relative cardio-activities, so this system would be useful in therapeutic drug monitoring of digoxin.

Key words digoxin; anti-digoxin antiserum; radioimmunoassay (RIA); digoxin 3'-hemisuccinate–bovine serum albumin conjugate; digoxin 3''-hemisuccinyl- ^3H -leucine

Digoxin is the most commonly used digitalis glycoside for the treatment of congestive heart failure and atrial fibrillation. It is important to measure digoxin in serum from digitalized patients because the therapeutic range of serum digoxin concentration is narrow. Immunoassays are routinely used for therapeutic drug monitoring (TDM) of digoxin. The antibody with cross-reactivity to metabolites of digoxin (digoxigenin bis and monodigitoxosides, digoxigenin, and dihydrodigoxin) matching their relative cardio-activities should be used, and a good correlation must exist between the assay value and the pharmacological effect to avoid toxic dosage in the TDM of digoxin.^{1–5)} A receptor radioligand assay, using semi-purified Na^+/K^+ -ATPase from a human heart, and a commercial immunoassay, the ACSTM Digoxin assay, using a monoclonal anti-digoxin antibody, satisfied almost all these conditions.^{2,4,5)}

We recently reported the preparation of digoxin C-3' and C-3'' hemisuccinate–bovine serum albumin (BSA) conjugates, the former of which was then used for generating a new anti-digoxin antiserum. Digoxin concentration in serum from digitalized patients was measured by the radioimmunoassay (RIA) using this antiserum.^{6–8)} In the present study, we prepared new anti-digoxin antisera against the BSA conjugates as well as new labeled antigens in which the C-3' or C-3'' hemisuccinate derivative of digoxin was coupled with a ^3H -leucine. RIA was carried out in homologous and site heterologous combinations between the antiserum and labeled antigen, and one of these systems was found to have a desirable recognition pattern for digoxin metabolites that matches the metabolite pharmacological activity.

MATERIALS AND METHODS

Materials L-[3,4,5- ^3H (N)]-Leucine (5550 GBq/mmol and

6364 GBq/mmol) was supplied by New England Nuclear (Boston, MA, U.S.A.). The scintillation solution (Atomlight) was obtained from Packard BioScience B. V. (Groningen, The Netherlands), dihydrodigoxin from Boehringer Mannheim (Mannheim, Germany), 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 plus 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.⁹⁾

A dextran-coated charcoal suspension was prepared by continuously stirring Norit SX plus (500 mg) and Dextran T-70 (50 mg) in cold phosphate saline buffer (40 ml) for 10 min prior to use.

The antisera (I-1), (I-2), and (I-3) were prepared by immunizing rabbits with digoxin 3'-hemisuccinate–BSA conjugate and the antisera (II-1), (II-2), and (II-3) by digoxin 3''-hemisuccinate–BSA conjugate.⁷⁾ The commercial anti-digoxin BSA antiserum (III) was supplied by BioMakor Co. (Rehovot, Israel). Digoxin 3'-hemisuccinyl- ^3H -leucine (5550 GBq/mmol) (7) was prepared by a previously reported method.⁸⁾

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. ^1H -NMR spectra were recorded using tetra-

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methylsilane as an internal standard for a JEOL ECP-500 spectrometer at 500 MHz. Abbreviations used: s=singlet, d=doublet, and m=multiplet.

Digoxin 3''-Hemisuccinylleucine (6) Digoxin 3''-hemisuccinate *p*-nitrophenyl ester (**4**) was prepared from digoxin 3''-hemisuccinate (**3**).⁷ A solution of L-leucine (87 mg, 0.66 mmol) in H₂O (5 ml) was added to a solution of **4** (140 mg, 0.13 mmol) in pyridine (5 ml), and the mixture was stirred at room temperature for 18 h. The solution was extracted with AcOEt, and the aqueous layer was percolated through an Amberlite XAD-2 column (45×1.5 cm i.d.). The column was washed with H₂O, and then the desired compound was eluted with MeOH. After evaporation of the MeOH fraction, the crude product obtained was submitted to silica-gel column (93×1.3 cm i.d.) chromatography using CHCl₃-MeOH-H₂O (80:20:2.5, v/v/v) as a mobile phase and further purified on a Sephadex LH-20 column (45×1.8 cm i.d.) using MeOH as an eluent. The eluate was recrystallized from acetone-hexane to give **6** (41 mg, 32%) as a colorless amorphous solid. mp 172–175 °C. $[\alpha]_D^{25} +35.9^\circ$ ($c=0.12$, MeOH). Anal. Calcd for C₅₁H₇₉NO₁₈·1/2H₂O: C, 61.06; H, 8.04; N, 1.40. Found: C, 61.05; H, 7.84; N, 1.42. FAB-MS m/z : 1016 [M+Na]⁺. UV λ_{max} (MeOH) nm (ϵ): 217 (13800). ¹H-NMR (CD₃OD) δ : 0.78 (3H, s, 18-CH₃), 0.92–0.95 (9H, m, 19- and leucine-CH₃), 1.18–1.20 (9H, unresolved d, digitoxose-CH₃), 2.60 (4H, s, -CO(CH₂)₂CO-), 4.91 (2H, m, 21-CH₂), 5.33 (1H, m, 3''-H), 5.90 (1H, s, 22-H).

Digoxin 3''-Hemisuccinyl-[³H]-leucine (8) A solution of [³H]-leucine (1.0 μ Ci, 6364 GBq/mmol) in EtOH-H₂O (2:98, v/v) (1.0 ml) was added to a solution of **4** (8.0 mg) in pyridine (1.0 ml), and the mixture was stirred at room temperature for 4 h. After addition of H₂O (10 ml), the reaction mixture was passed through a Sep-Pak C₁₈ cartridge, and after washing with 30% MeOH (50 ml), MeOH (5 ml) was passed through the cartridge. The MeOH fraction was evaporated and dissolved in CHCl₃-MeOH-H₂O (90:10:0.8, v/v/v) (2 ml), and loaded on a Sep-Pak silica cartridge. After washing with CHCl₃-MeOH-H₂O (90:10:0.8, v/v/v) (48 ml), **8** was eluted with CHCl₃-MeOH-H₂O (85:15:1, v/v/v) (50 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 RIA was performed in phosphate saline buffer (pH 7.4) containing K₂HPO₄ (0.696 g), NaH₂PO₄·H₂O (0.138 g), NaCl (4.39 g), and BSA (1.0 g) in H₂O (500 ml). For the digoxin free serum, a sample pool serum from healthy volunteers who had not taken any drugs was used. The digoxin free serum (0.2 ml), tracer (*ca.* 12000 dpm) in assay buffer (0.1 ml), and diluted antiserum (0.3 ml) were added to tubes containing from 0 to 10 ng of 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 vortex-mixed, 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, a scintillation solution (6 ml) was added, and the radioactivity was counted in an Aloka LSC-6000 liquid scintillation counter. The radioactivity bound to the antibody was calculated after correction for the blank value measured using the assay buffer instead of the antiserum.

Cross-Reaction Study The specificities of the antisera were tested by calculating the percentage of cross-reactivity with digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, digoxigenin, and dihydrodigoxin.^{10,11} Cross-reactivity was determined by the above-mentioned assay procedure, by comparing the concentrations of digoxin and test compounds necessary for a 50% displacement of the antibody-bound tracer.

RESULTS AND DISCUSSION

The sensitivity of RIA increases when a tracer with a higher specific radioactivity is used. Thus, a ¹²⁵I-labeled antigen is expected to provide a higher sensitivity than the corresponding ³H-labeled antigen. Although ¹²⁵I-labeled digoxin derivatives have been used for analyzing digoxin in biological fluids, the ¹²⁵I-labeled compounds have disadvantages due to their much shorter half-life.^{12–14} Oliver *et al.* used ¹²⁵I-labeled tyrosine-digitoxigenin 3-hemisuccinate for measuring digitoxin in serum.¹⁵ The design of this tracer was applied to ³H-labeled antigen in the present study. The synthesis of digoxin 3'- and 3''-hemisuccinyl-[³H]-leucine (**7** or **8**) (Chart 1) was carried with ³H-labeled amino acid, L-[3,4,5-³H(N)]-leucine which has relatively high specific radioactivity. The radiochemical purity of the tracers was checked by TLC, using a previously reported method.⁸ Digoxin 3''-hemisuccinylleucine (**6**) was prepared from digoxin 3''-hemisuccinate *p*-nitrophenyl ester (**4**), which was derived from **3** and used as a standard on TLC.

The properties of the antibodies are shown in Table 1. The final dilutions were defined to bind approximately 50% of the tracer and the association constants were determined from a Scatchard plot.¹⁶ When digoxin 3'-hemisuccinyl-[³H]-leucine, which is the homologous antigen, was used, the sensitivities of the antisera (I) ((I-1), (I-2), (I-3)) elicited by digoxin 3'-hemisuccinate-BSA conjugate were higher than when digoxin 3''-hemisuccinyl-[³H]-leucine, the site heterologous antigen was used. Also, these factors of the antisera (II) ((II-1), (II-2), (II-3)) elicited by digoxin 3''-hemisuccinate-BSA conjugate showed high potencies with use of the

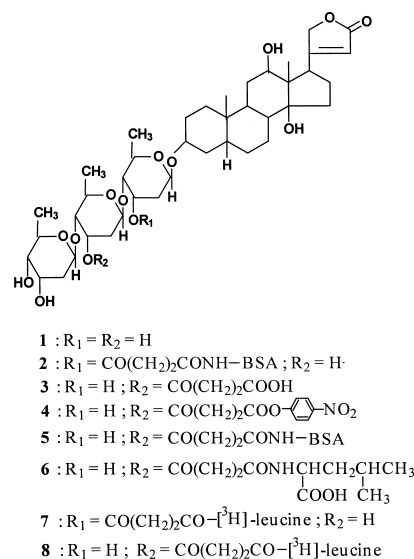


Chart 1

Table 1. Final Dilutions, Association Constants, and Ranges of the Standard Curves for Digoxin Using Anti-digoxin Antiserum

Digoxin 3'-hemisuccinyl-[³ H]-leucine							
	Antiserum (I-1)	Antiserum (I-2)	Antiserum (I-3)	Antiserum (II-1)	Antiserum (II-2)	Antiserum (II-3)	Antiserum (III)
Final dilution	1 : 31000	1 : 4700	1 : 12000	1 : 4600	1 : 19000	1 : 240	1 : 31000
Association constant (M^{-1})	1.1×10^9	3.8×10^8	2.8×10^8	6.1×10^8	2.1×10^8	7.5×10^7	1.0×10^9
Range (ng)	0.03—1.5	0.03—1.5	0.03—3.0	0.2—0.3	0.05—1.0	0.5—10	0.02—0.7
Digoxin 3''-hemisuccinyl-[³ H]-leucine							
Final dilution	1 : 1800	1 : 1100	1 : 2800	1 : 22000	1 : 7000	1 : 2400	1 : 16000
Association constant (M^{-1})	7.9×10^7	8.2×10^7	3.7×10^8	3.6×10^8	7.8×10^8	1.0×10^8	2.3×10^8
Range (ng)	0.15—3.0	0.15—3.0	0.15—3.0	0.03—1.5	0.03—1.5	0.1—3.0	0.03—1.5

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

Compound	% Cross-reactivity (50%)a						
	Digoxin 3'-hemisuccinyl-[³ H]-leucine						
	Antiserum (I-1)	Antiserum (I-2)	Antiserum (I-3)	Antiserum (II-1)	Antiserum (II-2)	Antiserum (II-3)	Antiserum (III)
Digoxin	100	100	100	100	100	100	100
Digoxigenin bisdigitoxoside	0.34	26	76	111	111	109	113
Digoxigenin monodigitoxoside	0.11	8.1	65	194	122	87	126
Digoxigenin	0.02	3.4	26	113	95	57	81
Dihydrodigoxin	9.4	6.8	1.2	17	0.41	0.47	0.77
Compound	Digoxin 3''-hemisuccinyl-[³ H]-leucine						
	Antiserum (I-1)	Antiserum (I-2)	Antiserum (I-3)	Antiserum (II-1)	Antiserum (II-2)	Antiserum (II-3)	Antiserum (III)
	Antiserum (I-1)	Antiserum (I-2)	Antiserum (I-3)	Antiserum (II-1)	Antiserum (II-2)	Antiserum (II-3)	Antiserum (III)
Digoxin	100	100	100	100	100	100	100
Digoxigenin bisdigitoxoside	108	87	84	44	83	37	83
Digoxigenin monodigitoxoside	112	76	82	1.7	91	20	67
Digoxigenin	67	54	64	<0.01	86	1.3	54
Dihydrodigoxin	0.77	0.75	0.74	85	0.65	17	0.56

a) Values are calculated on a molar basis.

homologous antigen, except for the final dilution of antiserum (II-2). When the homologous antigen is used, low digoxin concentration levels can be measured.

The specificities of anti-digoxin antisera were assessed by cross-reaction tests with digoxin metabolites. Percentages indicating cross-reactivity at 50% displacement of the antibody-bound tracer compared to digoxin were calculated, and the results are listed in Table 2. When the tracer with [³H]-leucine at the C-3' position was used, antisera (I-1) had no significant cross-reaction with digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, or digoxigenin. In contrast, the antisera (II) showed high cross-reactivity with metabolites formed by the successive cleavage of the digitoxose residues. On the other hand, with digoxin 3''-hemisuccinyl-[³H]-leucine, the antisera (II) were able to recognize the sugar moiety and the antisera (I) were inferior in terms of specificity. Antiserum (III) was a commercial antiserum produced by immunization with a hapten carrier protein conjugate, which was coupled to the terminal digitoxose of digoxin using periodate oxidation.^{17,18} When the tracer used digoxin 3''-hemisuccinyl-[³H]-leucine, the specificities of antiserum (III) to the digitoxose chain were better than when digoxin 3'-hemisuccinyl-[³H]-leucine was used. Because the digitoxose C-3'' position is near the terminal digitoxose, the

specificity of the anti-digoxin antiserum might have been improved to recognize the sugar moiety. A [³H]-digoxin was used with digoxin RIA to report the properties of antiserum (I-1) and (II-2).⁷ The cross-reactivity pattern using [³H]-digoxin was similar to the homologous RIA system in the present study. However, the RIA using [³H]-digoxin still lacks the sensitivity needed for measurement of digoxin concentration in serum, though it may be useful to investigate the specificity of anti-digoxin antiserum.

Immunoassays for cardiac glycosides are routinely used for TDM. It has been suggested that immunoassays for digoxin that cross-react to active metabolites are more comparable to the total biological activities of the drug, and a good correlation must exist between the assay value and the pharmacological effect. This RIA system using antiserum (I-3) with digoxin 3'-hemisuccinyl-[³H]-leucine meets this standard. Antiserum (I-3) showed digoxigenin bisdigitoxoside (76%), digoxigenin monodigitoxoside (65%), digoxigenin (26%), and dihydrodigoxin (1.2%). These cross-reactivity results agreed with the cardioactivity of digoxigenin bisdigitoxoside (77%), digoxigenin monodigitoxoside (66%), digoxigenin (4—21%), and dihydrodigoxin (2—6%).^{19—21} The standard curve obtained with this RIA system is presented in Fig. 1. The plot of logit percent bound radioactivity

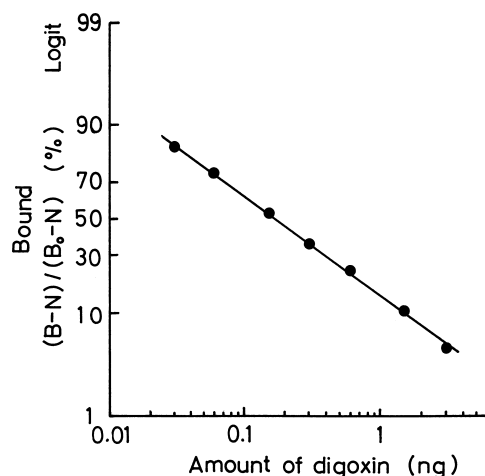


Fig. 1. Standard Curve for Digoxin by the Digoxin RIA System Using Antiserum (I-3) with the Homologous Antigen

vs. logarithm of the amount of digoxin showed a linear relationship over the range of 0.03 to 3.0 ng.

This study demonstrated that the RIA system using antiserum (I-3) with the homologous antigen would be useful in TDM of digoxin. The pharmacokinetics and TDM studies of digoxin may utilize novel anti-digoxin antisera. It was also shown that specificity of an antiserum can vary by combining a labeled-antigen having a different chemical structure.

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