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Research Article

Establishment of a Highly Specific Enzyme Immunoassay for Digoxin in Human Serum

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Abstract

We previously developed a radioimmunoassay for digoxin using an antiserum raised against digoxin 3'-hemisuccinate -bovine serum albumin. In the present study, we aimed to establish an enzyme immunoassay (EIA) for measuring serum digoxin level in patients, using digoxin 3'-hemisuccinate-β-D-galactosidase as an enzyme-labeled antigen. The developed enzyme immunoassay showed a quantification range of 0.2 to 10 ng/ml and exhibited high specificity for digoxin, with low levels of cross-reaction to dihydrodigoxin (12.3%), digitoxin (7.84%), digoxigenin bisdigitoxoside (0.31%), digoxigenin monodigitoxoside (0.19%), and digoxigenin (0.05%). Compared with a commercial anti-digoxin antiserum clinically used to monitor digoxin concentration in human serum, our antiserum showed much higher specificity for intact digoxin. Intra-assay and inter-assay variations were less than 9.3% and 8.8%, respectively. Recovery was within the range of 96.0 -105.0%. Mean digoxin concentrations measured in serum samples (n=42) from digoxin-treated patients by EIA using the new antiserum and the commercial anti-digoxin antiserum were 1.26 and 1.39 ng/ml, respectively. The present EIA, which is superior to RIA in terms of convenience and disposal of waste materials, is expected to be practically useful to monitor intact digoxin in human serum.

Keywords: Digoxin; Digoxin 3'-hemisuccinate-β-D-galactosidase; Digoxin 3'-hemisuccinate-bovine serum albumin; Antiserum; Enzyme immunoassay; Cross-reactivity

Abbreviations

EIA: Enzyme Immunoassay;
BSA: Bovine serum albumin;
RIA: Radioimmunoassay;
Antiserum-A: Antiserum raised against digoxin 3'-hemisuccinate-BSA;
Antiserum-B: Commercially available anti-digoxin antiserum

Introduction

Digoxin is clinically used to treat congestive heart failure and atrial fibrillation. However, therapeutic monitoring is important, because the effective concentration range in serum is very narrow. Immunoassay procedures have been developed to monitor the concentration of cardiac glycosides in serum, using antisera produced by immunization with hapten-carrier protein conjugates formed by periodate-mediated oxidative coupling at the terminal digitoxose of digoxin [1,2]. However, because administered digoxin is metabolized to digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, digoxigenin, and dihydrodigoxin in the body (Chart 1) [3-5], the specificity of these assays for intact digoxin is relatively poor.

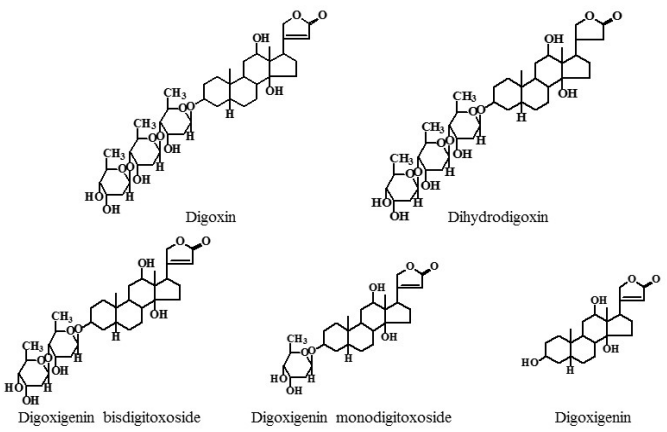


Chart 1. Chemical structures of digoxin and its metabolites.

To obtain a highly specific antibody, the binding position of the hapten to the carrier protein is critical. Shimada *et al.* [6] and Thong *et al.* [7] used conjugates in which the hapten was linked to the carrier protein at the C-12 (or C-17) and C-22 positions, respectively. However, these antisera exhibited high cross-reactivity with dihydrodigoxin, one of the metabolites of digoxin. We have reported the preparation and antigenic properties of digoxin-bovine serum albumin (BSA) and digitoxin-BSA conjugates linked at the digitoxose C-3' and C-3'' positions in radioimmunoassay (RIA) [8-11]. The antiserum raised against digoxin 3'-hemisuccinate-BSA (Antiserum-A) showed much higher specificity for intact digoxin than did commercially available anti-digoxin antiserum (Antiserum-B), and was applied for pharmacokinetic studies of digoxin in rats [12]. In addition, we developed an RIA using a novel antigen, ^{3}H-labelled leucine-digoxin 3'-hemisuccinate [13,14]. However, RIA requires special facilities and involves waste disposal problems, so a simpler, more convenient method is still required.

Electrochemical immunosensors for digoxin have been developed. Omidfer *et al.* [15] established a semi-quantitative immunostrip assay for digoxin using an antibody–gold nanoparticle conjugate. However, it showed low sensitivity and proved unreliable for quantitative analysis in biological samples. An electrochemical immunosensor for digoxin using core-shell gold-coated magnetic nanoparticles was also proposed, but the specificity of the antibody for digoxin metabolites was not investigated [16]. Therefore, this method might also be unsuitable for quantitation of digoxin levels in biological samples. In addition, a simultaneous quantification of digoxin, digitoxin, and their metabolites in serum by means of LC/MS was recently developed [17]. This method avoids the possibility of interference by digoxin metabolites, but is relatively complicated and expensive.

Up to now, the specificities of commercial antibody were investigated, and analytical interference due to other drugs was reviewed in digoxin immunoassays [18,19]. The information received from these results indicate that the much lower cross-reactivity of the antibody will be very important. Improvement of antibody on immunoassay technique of digoxin will be more suitable for pharmacokinetic studies and therapeutic monitoring for patients. Here, we describe the preparation of digoxin 3'-hemisuccinate-*β*-D-galactosidase as an enzyme-labeled antigen from digoxin 3'-hemisuccinate *p*-nitrophenyl ester, and its application to establish an enzyme immunoassay (EIA) for digoxin. We confirmed that EIA using our Antiserum-A is superior to EIA using commercial Antiserum-B in terms of specificity for intact digoxin, and we compared digoxin levels measured with the two EIAs in serum of digoxin-treated patients.

Material and Methods

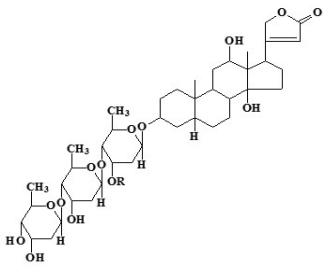
Materials

Digoxin was obtained from Aldrich (Milwaukee, WI, U.S.A.). Spironolactone, BSA (fraction V), anti-rabbit IgG antiserum raised in goat, and anti-digoxin antiserum were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and dihydrodigoxin was from Boehringer Mannheim (Mannheim, Germany). Sephadex G-25 and G-100 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Digitoxin, *β*-D-galactosidase (EC 3.2.1.23) from *Escherichia coli*, 4-methylumbelliferyl-*β*-D-galactopyranoside, and general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). Digoxigenin, digoxigenin monodigitoxoside, and digoxigenin bisdigitoxoside were prepared by hydrolysis of digoxin according to the methods of Kaiser and co-workers [20].

Preparation of *β*-D-galactosidase-labeled antigen (Chart 2)

Digoxin 3'-hemisuccinate *p*-nitrophenyl ester was prepared

from digoxin 3'-hemisuccinate, according to our previous work [13]. Conjugation of β-D-galactosidase was carried out in the same manner as described previously in connection with our establishment of an EIA for β-methyldigoxin using anti-β-methyldigoxin 3'-hemisuccinate-BSA antiserum [21].



R= $CO(CH_2)_2COOH$, digoxin 3'-hemisuccinate
R= $CO(CH_2)_2COO$—NO_2, digoxin 3'-hemisuccinate *p*-nitrophenyl ester
R= $CO(CH_2)_2CONH$-β-D-galactosidase, digoxin 3'-hemisuccinate—β-D-galactosidase

Chart 2. Synthesis of digoxin 3'-hemisuccinate—β-D-galactosidase.

A solution of digoxin 3'-hemisuccinate *p*-nitrophenyl ester (6.3 mg) in dioxane (1.5 ml) were incubated with a solution of β-D-galactosidase (2.1 mg) in 1 ml of 0.05 M phosphate buffer (pH 9.0) for 4 h at 4°C. The reaction mixture was chromatographed on a Sephadex G-25 column (45×1.5 cm i.d.) with 0.05 M phosphate buffer (pH 7.0) as the mobile phase. Fractions (each 2.5 ml) representing the main peak of the enzyme activity were combined and further subjected to Sephadex G-100 column (45×1.5 cm i.d.) chromatography with 0.05 mol/l phosphate buffer (pH 7.0) as the mobile phase. Fractions representing the main peak of the enzyme activity were used as the labeled antigen in EIA. The presence of steroid structure was confirmed by TLC plates with $CHCl_3$-MeOH-AcOH (90:10:0.8, v/v), using concentrated H_2SO_4 spray for detection. The results confirmed the formation of enzyme-labeled antigen (digoxin 3'-hemisuccinate-β-D-galactosidase).

EIA procedure

EIA is based on the principle of competition between enzyme-labeled and unlabeled drugs for an antibody, followed by measurement of the marker enzyme activity of the immunoprecipitate. Phosphate-buffered saline for EIA was adjusted to pH 7.3 by addition of a solution containing NaH_2PO_4 (3.9 g), NaCl (4.5 g), NaN_3 (0.5 g), $MgCl_2$ (0.005 g), and BSA (0.5 g) in H_2O (500 ml) to a solution containing K_2HPO_4 (8.7 g), NaCl (9.0 g), NaN_3 (1.0 g), $MgCl_2$ (0.01 g), and BSA (1.0 g) in H_2O (1000 ml). An aliquot of 50 µL of digoxin (0.8 to 80 ng/ml) in phosphate buffer was added to drug-free serum (0.2 ml, serum digoxin concentration of 0.02 to 20 ng/ml). An aliquot of 50 µl of digoxin-free phosphate buffer was added to an aliquot of patient's serum (0.2 ml). These mixtures were used for assay. Each sample (250 µl) and diluted Antiserum-A (0.1 ml, 1:2400) or Antiserum-B (0.1 ml, 1:1200) were mixed and incubated for 2.5 h at 4°C. Then, 0.1 ml of synthetic enzyme-labeled antigen (diluted 1:1200 in phosphate-buffered saline) was added and the mixture was incubated for 0.5 h at 4°C. An aliquot of 0.2 ml of goat anti-rabbit IgG antiserum (1.67%) and 0.1 ml of normal rabbit serum (1%) were added and the mixture was left to stand for 12 h at 4°C, and then centrifuged at 1600×*g* for 20 min. The supernatant was aspirated, and the immunoprecipitate was washed twice with 1 ml of phosphate-buffered saline. The activity of enzyme conjugate bound to each tube was measured by the addition of 0.5 ml of 4-methylumbelliferyl-β-D-galactopyranoside (2.0×10^{-5} mol/l), followed by incubation of the tubes at 30°C for 3 h after pre-incubation with 0.5 ml of phosphate-buffered saline at 30°C for 3 min. The enzyme reaction was stopped by addition of 2.0 ml of glycine-NaOH buffer (0.1 mol/l, pH 10.3) to each tube, and the resulting 4-methylumbelliferone was measured by spectrofluorometry at wavelengths of 362 nm for excitation and 446 nm for emission, using a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan).

Cross-reaction study

The specificities of Antiserum-A and Antiserum-B were tested by calculating the percentage cross-reactivity with various compounds. Cross-reactivity was determined using the above-mentioned assay procedure, by comparing the concentrations of digoxin and test compounds necessary for 50% displacement of the antibody-bound enzyme-labeled digoxin.

Results and Discussion

Standard Curve

The standard curve of digoxin using homologous assay with Antiserum-A is presented in Figure 1. The plot of percent bound fluorescence intensity *vs.* logarithm of the concentration of non-labeled digoxin showed a quantification range of 0.2 to 10 ng/ml. On the other hand, the standard curve of digoxin using Antiserum-B gave a narrower quantification range of 0.5 to 5 ng/ml. Thus, Antiserum-A is superior to Antiserum-B in terms of quantification range and limit.

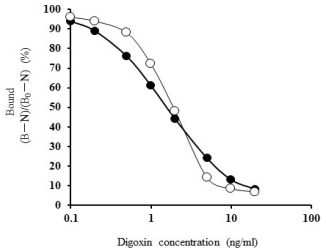


Figure 1. Standard curves of digoxin obtained by EIA using anti-digoxin 3'-hemisuccinate—BSA antiserum (Antiserum-A) and commercially available anti-digoxin antiserum (Antiserum-B). Antiserum-A (●), Antiserum-B (○).

Table 1. Cross-reaction data for EIA with Antiserum-A and Antiserum-B, and our previous data by RIA

	% Cross-reactivity (50%)					
	EIA		RIA-1 [12]		RIA-2 [13]	
	Antiserum-A	Antiserum-B	Antiserum-A	Antiserum-B	Antiserum-A	Antiserum-B
Digoxin	100	100	100	100	100	100
Digoxigenin bisdigitoxoside	0.31	124	0.95	126	0.34	111
Digoxigenin monodigitoxoside	0.19	86.7	1.2	132	0.11	122
Digoxigenin	0.05	60.1	0.23	103	<0.05	82.2
Dihydrodigoxin	12.3	4.20	3.3	4.7	9.4	0.80
Digitoxin	7.84	0.70	(-)	(-)	6.1	0.60
Spironolactone	<0.05	<0.05	(-)	(-)	<0.05	<0.05
Digitoxose	<0.05	<0.05	(-)	(-)	<0.05	<0.05
Progesterone	<0.05	<0.05	(-)	(-)	<0.05	<0.05
Testosterone	<0.05	<0.05	(-)	(-)	<0.05	<0.05
Cholesterol	<0.05	<0.05	(-)	(-)	<0.05	<0.05

Values are calculated on a molar basis.

Data from our previous papers, RIA-1: [^{3}H]digoxin as a labeled antigen [12], RIA-2: digoxin 3'-hemisuccinate-[^{3}H]leucine as a labeled antigen [13].

(-), not investigated.

Cross-Reactivity

The specificities of Antiserum-A and Antiserum-B were assessed by means of cross-reaction assays with various related compounds. The percentage cross-reactivity was calculated at 50% displacement of the antibody-bound labeled digoxin, and the results are listed in Table 1. Antiserum-A showed high specificity for digoxin, exhibiting low cross-reactions with dihydrodigoxin (12.3%) and digitoxin (7.84%). Further, there was little cross-reaction with digoxigenin bisdigitoxoside (0.31%), digoxigenin bisdigitoxoside (0.19%), or digoxigenin (0.05%). In contrast, Antiserum-B exhibited considerable cross-reactivity with digoxigenin bisdigitoxoside (124%), digoxigenin monodigitoxoside (86.7%), and digoxigenin (60.1%), although it showed low cross-reaction with dihydrodigoxin (4.20%), and had no significant cross-reaction with digitoxin (0.70%). Other compounds tested showed negligible values of cross-reactivity of < 0.05% with both antisera. Thus, Antiserum-B showed high cross-reactivity with metabolites formed by successive cleavage of digitoxose residues, being greatly inferior to Antiserum-A for monitoring of intact digoxin. This result may reflect the production of Antiserum-B by using peroxidate-oxidized an antigen [5]. In our previously developed RIA, Antiserum-A also showed high specificity, exhibiting low or no significant cross-reaction with various digoxin metabolites. In contrast, Antiserum-B possessed much lower specificity for digoxin, exhibiting high levels of cross-reaction with digoxin metabolites. A cross-reaction study using antiserum raised against a 12-dehydrodigoxin derivative-BSA conjugate showed considerable cross-reactions to dihydrodigoxin (65.3%) and digoxin (29.1%), but not digoxigenin bisdigitoxoside (0.6%), digoxigenin monodigitoxoside (0.2%) or digoxigenin (< 0.01%) [6]. Tong *et al.* [7] prepared digoxin-BSA conjugate *via* reductive ozonolysis of the lactone ring, and observed considerable cross-reactivities for dihydrodigoxin (67%), digoxigenin bisdigitoxoside (14.6%), and digitoxin (11%), though cross-reactivity for digoxigenin monodigitoxoside was low (3.7%). In the present EIA, the high specificity of our Antiserum-A in RIA was well retained.

Table 2. Recovery of digoxin by EIA using Antiserum-A

Intra-assay (n=7)			
Added (ng/ml)	Measured (ng/ml)	C.V. (%)	Recovery (%)
	Mean ± S.D.		
0.5	0.48 ± 0.04	8.7	96.0
1	1.05 ± 0.07	6.9	105.0
2	1.95 ± 0.11	5.5	97.5
5	4.92 ± 0.46	9.3	98.4
Inter-assay (n=7)			
Added (ng/ml)	Measured (ng/ml)	C.V. (%)	Recovery (%)
	Mean ± S.D.		
0.5	0.51 ± 0.05	8.8	102.0
1	1.01 ± 0.08	7.5	101.0
2	1.99 ± 0.14	7.0	99.5
5	4.99 ± 0.31	6.3	99.8

Validation of EIA

As shown in Table 2, to investigate the precision of the EIA, we examined intra- and inter-assay reproducibility (n=7) in human serum spiked at 0.5, 1, 2, and 5 ng/ml by homologous assay using Antiserum-A, which showed the highest selectivity for digoxin. The intra-assay coefficient of variation was in the range of 6.9 to 9.3%, and the recovery was 96.0 to 105.0%. In the inter-assay study, the range of standard deviation was 6.3-8.8%, and the recovery was 99.5-102.0%. These data show that the EIA using digoxin 3'-hemisuccinate-β-D-galactosidase and Antiserum-A gave satisfactory precision and accuracy.

Table 3. Comparison of interference by digoxin metabolites in EIA of digoxin using Antiserum-A and Antiserum-B

Added	Antiserum-A			Antiserum-B		
	Measured (ng/ml) Mean ± S.D. (n=7)	C.V. (%)	Recovery (%)	Measured (ng/ml) Mean ± S.D. (n=7)	C.V. (%)	Recovery (%)
Digoxin (0.5 ng/ml)						
+Metabolites (each 0.05 ng/ml)	0.50 ± 0.03	6.0	100.0	0.78 ± 0.07	9.0	156.0
Digoxin (1 ng/ml)						
+Metabolites (each 0.1 ng/ml)	1.05 ± 0.10	9.5	105.0	1.25 ± 0.12	9.6	125.0
Digoxin (2 ng/ml)						
+Metabolites (each 0.2 ng/ml)	2.12 ± 0.11	5.2	106.0	2.55 ± 0.15	5.9	127.5

Metabolites: digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, digoxigenin, and dihydrodigoxin.

Interference by digoxin metabolites

As shown in Table 3, we investigated whether digoxin metabolites interfered with the assay in human serum. Each metabolite was added to human serum at a ratio of 10% to digoxin, and a recovery test was performed. The recovery ratios using Antiserum-A at 0.5, 1, and 2 ng/ml were 100.0%, 105.0%, and 106.0%, respectively. In contrast, those using Antiserum-B at 0.5, 1, and 2 ng/ml were 156.0%, 125.0%, and 127.5%, respectively. These results indicate that the interference by digoxin metabolites was significant in the case of Antiserum-B, but was greatly reduced with Antiserum-A.

Digoxin concentration in serum of digoxin-treated patients

Serum samples (n=42) obtained from digoxin-treated patients were examined by EIA using Antiserum-A, and the assay performance was compared with that of EIA using Antiserum-B (Figure 2). When Antiserum-A was used, the mean digoxin concentration was obtained as 1.26 ng/ml (range, 0.36 to 2.85 ng/ml). When the same samples were measured with Antiserum-B, the mean value of digoxin was 1.39 ng/ml (range, 0.40 to 2.86 ng/ml). The ratio of concentrations obtained by EIA using Antiserum-A to those using Antiserum-B was 0.9232. We consider that the values obtained using Antiserum-B were higher than those obtained using Antiserum-A because of the greater extent of cross-reaction of Antiserum-B with digoxin metabolites, *i.e.*, Antiserum-A measures predominantly intact digoxin, while Antiserum-B measures both intact digoxin and its metabolites. The results also suggest that there are marked inter-individual differences between patients with regard to metabolism of digoxin to form digoxigenin, its mono- and bisdigitoxosides and dihydrodigoxin.

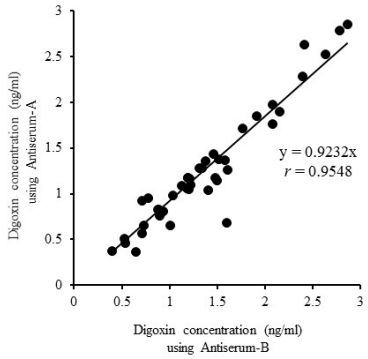


Figure 2. Comparison of digoxin concentrations in serum from digoxin-treated patients, measured by EIA using Antiserum-A and Antiserum-B.

Conclusion

We established and validated an EIA with high specificity for digoxin over its metabolites by using digoxin 3'-hemisuccinate-β-D-galactosidase as an enzyme-labeled antigen and Antiserum-A as a specific antiserum, based on our previously established RIA for digoxin. The developed EIA measures intact digoxin with high specificity, and should be suitable for pharmacokinetic studies and therapeutic monitoring. Antiserum-A may also be useful for treatment of digoxin overdose.

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