Digoxin Immunoassay with Monoclonal and Polyclonal Antibodies Using Time-Resolved Fluorometry

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Received October 17, 1988, from *CyberFluor, Inc., 179 John Street, Toronto, Ontario, M5T 1X4, Canada, and the ‡Department of Clinical Biochemistry, University of Toronto, 100 College Street, Toronto, Ontario, M5G 1L5, Canada. Accepted for publication January 18, 1989.

Abstract □ We describe a nonisotopic heterogeneous competitive immunoassay of digoxin in serum using either Fab fragments of a polyclonal antibody or a high-affinity monoclonal antibody. In the assay, digoxin competes with immobilized digoxin (digoxin:thyroglobulin conjugate) for binding to a biotinylated immunoreactant (Fab or monoclonal). The amount of biotinylated moiety bound to the solid phase (white polystyrene microtiter wells), which is inversely related to the amount of digoxin in the sample, is then quantified by adding streptavidin labeled with the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) in the presence of excess Eu^{3+}. The fluorescent immunocomplex formed is measured directly on the dry solid phase by time-resolved fluorometry. The assay is simple to perform and its characteristics are similar to those of other currently used immunoassay techniques. The Fab fragments and the monoclonal antibody procedure performed equally well on the system. Our results suggest that monoclonal antibodies with high affinity for digoxin can be used for the routine determination of the drug in serum.

Digoxin is the most widely used cardiac glycoside for the control of congestive heart failure and certain abnormalities of cardiac rhythm.[1] The therapeutic use of this drug is constrained by its narrow therapeutic range.[2] Levels in serum >2.5 μg/L are usually associated with toxic effects. To avoid toxicity during the initial digitalizing dose or the subsequent maintenance doses, it is imperative to monitor the serum levels of the drug. The original methods of measuring digoxin in serum, based on either the inhibition of rubidium uptake by erythrocytes[3,4] or sodium-potassium ATPase inhibition[5] by the drug, were replaced by sensitive radioimmunoassays introduced by Smith et al. in 1969.[6] Although radioimmunoassays are still widely used,[7,8] the recent trend is to replace the radionuclide-based tracers with alternative labels to avoid the well-known disadvantages of radioactivity.[9] Currently, fluorescence immunoassays[10–12] and enzyme immunoassays[13–19] are widely used because of their speed, simplicity, and good analytical performance.

Current digoxin immunoassays almost exclusively utilize high-affinity polyclonal antibodies. On the other hand, monoclonal antibodies are now being used in many immunoassays for large antigens because they exhibit a number of well-known advantages. Recently, high-affinity monoclonal antibodies for haptens have been produced and applied successfully for hapten immunoassays (e.g., cortisol,[20] thyroxine,[21] and triiodothyronine in serum). Mudgett-Hunter et al. produced many high-affinity digoxin murine monoclonal antibodies and applied them preliminarily in digoxin immunoassays.[22,23]

Recently, the fluorescent europium chelates have received much attention as potential immunological labels. These labels can be used in time-resolved fluorometric applications, thus eliminating many of the limitations of conventional fluorescent probes. Time-resolved fluorescence takes advantage of the extremely long fluorescence lifetime of the europium chelates and eliminates short-lived background fluorescence by carrying out measurements only during a preselected time window. Time-resolved fluorescence, the europium chelates, and their applications to immunoassays are discussed in detail in a number of recent reviews.[9,24]

Currently, the fluorescent europium chelates have been applied in immunoassays in two different assay configurations. In the first system,[24] Eu^{3+} is used as a label. In an alternative system proposed by our group, a europium chelator, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9 dicarboxylic acid (BCPDA) is used as a label.[9] The two systems have recently been reviewed and critically compared.[9] Helsingius et al. reported on a solid-phase immunoassay of digoxin using a polyclonal antibody labeled with Eu^{3+}.[25] In this paper, we describe a new time-resolved fluorometric immunoassay of digoxin using either monovalent Fab fragments of a rabbit antibody or a whole murine monoclonal antibody. In the assay, serum digoxin competes with immobilized digoxin (digoxin:thyroglobulin conjugate adsorbed on white microtitration wells) for binding to a soluble biotinylated Fab fragment of a polyclonal antibody or biotinylated whole monoclonal antibody. After washing, the degree of binding of the biotinylated moiety to the solid phase, which is inversely related to the digoxin concentration in the sample, is quantified by a bridge reaction with streptavidin which has been covalently linked to a bulking protein agent (thyroglobulin) carrying multiple BCPDA residues. In the presence of excess Eu^{3+}, the fluorescent complex on the dried solid phase is then measured in an automated time-resolved fluorometer. In this procedure, the major limitation of the previous time-resolved fluoroimmunoassay for digoxin,[25] the vulnerability to exogenous Eu^{3+} contamination,[9] has been eliminated by using BCPDA as a label and performing the assay in the presence of a saturating concentration of europium.

Experimental Section

Instrumentation—For solid-phase time-resolved fluorometric measurements, we used the Model 615 Immunoanalyzer available commercially through CyberFluor, Toronto, Canada. Radioactivity counting was performed with the LKB Wallac 1275 Minigamma counter.

Chemicals and Solutions—The europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesized as described by Evangelista et al.[26] Streptavidin and bovine serum albumin (BSA) were purchased from Sigma Chemical, St. Louis, MO, and $EuCl_3$ hexahydrate was from Aldrich Chemical, Milwaukee, WI. Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-Biotin) was from Pierce Chemical, Rockford, IL. All other chemicals were from Sigma. White opaque 12-well microtiter strips were products of Dynatech Labs, Alexandria, VA.

The coating buffer was a 0.1 mol/L Tris solution, pH 7.40. The blocking buffer was a 50 mmol/L sodium phosphate solution, pH 7.4, containing 9 g of NaCl, 10 g of bovine serum albumin (BSA), and 1 mL of polyoxyethylenesorbitan monolaurate (Tween 20) per liter. The digoxin assay buffer was a 50 mmol/L Tris-HCl solution, pH 7.8, containing 10 g of BSA, 0.5 g of bovine globulin, 9 g of NaCl, and 0.5

0022-3549/89/0800-0617$01.00/0

g of sodium azide per liter. The streptavidin:europium dilution buffer was 50 mmol/L Tris-HCl solution, pH 7.20, containing 9 g of NaCl, 40 g of BSA, 0.5 g of sodium azide, and 40 μmol of $EuCl_3$ per liter. The wash solution was a 9 g/L NaCl solution containing 0.5 mL of Tween 20 per liter.

Digoxin standards were prepared in normal human serum.

Comparative Methods—For comparison studies we used the RIANEN double-antibody radioimmunoassay kit from New England Nuclear, North Billerica, MA, and the automated TDx digoxin fluorescence polarization immunoassay from Abbott Diagnostics, Dallas, TX.

Antibodies—Rabbit anti-digoxin antiserum was purchased from Western Chemical Research, Ft. Collins, CO. Ascites fluid containing the monoclonal antibody used was obtained from Dr. Meredith Mudgett-Hunter. The characteristics of this antibody (Code Number 40-100) are described in detail elsewhere.[22,23]

Protein A Purification—Digoxin antibodies from antiserum and ascites fluid were isolated by using protein A affinity chromatography. All necessary reagents and columns were obtained in the form of a kit from Bio-Rad Chemical Division, Richmond, CA (Affi-Gel protein A MAPS II Kit). The instructions of the manufacturer were followed throughout. The isolated monoclonal antibody was extensively dialyzed against 5 L of a 9 g/L NaCl solution (changed three times) and then diluted to ~1 mg/mL (based on absorbance measurements at 280 nm). This antibody was then biotinylated as described below.

Preparation of Fab Fragments—The protein A-purified polyclonal rabbit anti-digoxin antibody was digested with agarose-immobilized papain and the Fc fragments were removed by passing through a protein A column. All reagents and columns for preparing Fab fragments were purchased in kit form from Pierce Chemical, Rockford, IL (ImmunoPure Fab Preparation Kit). The instructions from the manufacturer were followed throughout. The solution containing the Fab fragments was dialyzed extensively against 5 L of a 9 g/L NaCl solution (changed three times) and then concentrated to ~1 mg/mL (based on absorbance measurements at 280 nm) by using the Centricon 30 microconcentrators (from Amicon Canada, Oakville, Ontario, Canada).

Biotinylation—The monoclonal antibody and the Fab fragments were biotinylated as follows. One milliliter of solution (1 mg) was diluted 1:1 with a 0.5 mol/L carbonate buffer, pH 9.1. To this solution, 2 mg of NHS-LC-Biotin dissolved in 50 μL of dimethylsulfoxide were then added and the mixture was incubated for 2 h at room temperature. The solution was then dialyzed twice against 5 L of a 9 g/L NaCl solution at 4 °C. The biotinylated monoclonal antibody or the Fab fragments were then preliminarily diluted ~100-fold in the assay buffer and stored as such (they are stable for at least 1 year at 4 °C). For the assays, this solution was further diluted in the assay buffer to obtain the optimum working dilution (see below).

Tracer—The tracer used in this assay consists of the conjugate streptavidin:thyroglobulin:$(BCPDA)_{150}$. It is prepared by covalently linking streptavidin to bovine thyroglobulin that has been previously labeled with ~150 BCPDA molecules. The preparation of the tracer is described in detail elsewhere.[27] This tracer is stored as a 15 mg/L solution in terms of streptavidin content (stable at least 1 year at 4 °C) and is diluted 50-fold in the streptavidin:europium buffer just before the assay. The dilute tracer solution containing 0.30 mg/L streptavidin and 4×10^{-5} mol/L europium is stable for at least 4 h at room temperature.

Preparation of Digoxin:Thyroglobulin Conjugate—We prepared a digoxin:bovine thyroglobulin conjugate using the general method described by Butler and Tse-Eng,[28] but with certain modifications. In short, the procedure is as follows. Weigh 109.5 mg of digoxin (140 μmol) and dissolve it in 5 mL of ethanol. To this solution add, using continuous stirring, 5 mL of a 0.1 mol/L $NaIO_4$ solution and stir for an additional 30 min. Add 150 μL of a 1 mol/L ethylene glycol solution and incubate for 15 min with stirring (solution A). Dissolve 0.5 g of bovine thyroglobulin (0.76 μmol) in 10 mL of water and adjust the pH to 9.5 with a 5% (w/v) K_2CO_3 solution. To this solution add solution A (under continuous stirring) and incubate for 60 min. Add 5 mL of a fresh sodium borohydride solution (15 mg/mL) and leave overnight at room temperature. Adjust the pH to ~6.5 by the addition of a 1 mol/L formic acid solution and incubate for 1 h. Then, add 1 mol/L NH_4OH solution to raise the pH to 8.5. Dialyze the solution against running tap water for at least 48 h. Transfer the contents of the dialysis tubing to a beaker and add 0.1 mol/L HCl in a dropwise manner until precipitation is maximal (pH ~4.5). Transfer the contents to a centrifuge tube, incubate 1 h at room temperature and 3 h at 4 °C, and then centrifuge for 1 h at 4 °C (1000 × *g*). Discard the supernatant, dissolve the precipitate in 10 mL of a 0.15 mol/L $NaHCO_3$ solution, and dialyze again for 48 h against running tap water. Determine the protein content of the conjugate by using the Bio-Rad protein assay.

The stock digoxin:thyroglobulin conjugate was stored as a 20 mg/mL solution at 4 °C. An intermediate stock conjugate solution was also prepared with a concentration of 20 μg/mL. For coating, this intermediate stock was diluted further, 20-fold, in the coating buffer. Approximately 100 ng of conjugate was added per well during coating (see below).

Coating of Microtiter Strips—The strips were coated overnight at room temperature with 100 μL of a 1 μg/mL solution of digoxin:thyroglobulin conjugate in the coating buffer. After coating, the plates were rinsed once with the wash solution, blocked for 1 h at room temperature with 200 μL of the blocking buffer, washed twice, and air dried overnight at room temperature. When stored in sealed plastic bags at 4 °C, the coated strips were stable for at least 6 months.

Assay Procedure—Standard or serum samples (20 μL, in duplicates) were pipetted into each well and 100 μL of the working biotinylated Fab fragments or monoclonal antibody solution were added. The strips were then incubated at room temperature for 45 min by continuous shaking in an automatic shaking device. The strips were then washed four times with the wash solution and 100 μL of the working tracer reagent (streptavidin:thyroglobulin:BCPDA:Eu^{3+}) was added. After incubation for 30 min at 37 °C, the strips were washed four times with the wash solution and dried with a stream of air. Surface fluorescence was measured on the CyberFluor 615 Immunoanalyzer. The instrument has an automatic data reduction capability and results, along with the calibration curve, are printed automatically as soon as the readings are complete.

Results and Discussion

The principle of the assay design described here is shown in Figure 1. In the first step, digoxin present in the sample competes with immobilized digoxin for binding to a biotinylated immunoreactant (shown as a whole antibody in the figure). After washing out all unbound species, the tracer solution is added and incubated further in a second step. Excess tracer is washed out and the surface fluorescence is measured in a time-resolved mode. The resulting fluorescent immunocomplex is shown in Figure 1. After drying the well, this fluorescence remains on the solid phase for many months. The labeling of a streptavidin:thyroglobulin conjugate with BCPDA results in the incorporation of ~150 BCPDA molecules. This reagent is superior in terms of sensitivity to the one obtained with direct labeling of streptavidin with BCPDA because in the latter case only ~14 BCPDA molecules are incorporated.[27]

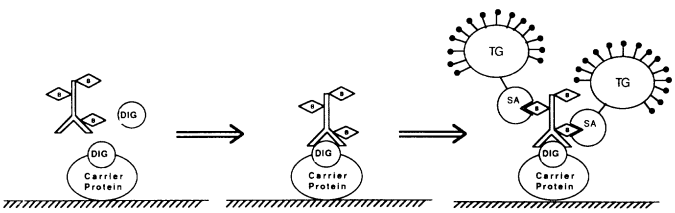

Figure 1—Schematic representation of the proposed digoxin assay (SA = streptavidin; B = biotin; carrier protein is thyroglobulin; —● is the BCPDA:Eu^{3+} complex; ≈≈ is the solid phase). The assay operates as follows. In the first incubation step, digoxin present in the sample (DIG) competes with immobilized digoxin (digoxin:thyroglobulin conjugate) for binding to a biotinylated immunoreactant (antibody or Fab fragments). After washing out all unbound species, the degree of binding of the biotinylated moiety is quantified by adding streptavidin covalently linked to BCPDA-labeled thyroglobulin, in the presence of excess Eu^{3+}. After washing and drying the solid phase, the final fluorescent immunocomplex (solid phase:thyroglobulin:digoxin:antibody:biotin:streptavidin:thyroglobulin:BCPDA:Eu^{3+}) is measured with the immunoanalyzer.

Assay optimization was done by studying all the factors that affect the shape of the standard curve, namely, the sample volume, the biotinylated antibody volume and concentration, the amount of immobilized digoxin conjugate, the incubation times, and the amount of tracer added. The sample and antibody volumes were chosen to be 20 and 100 μL, respectively. Coating with ~100 ng of digoxin conjugate resulted in sensitive calibration curves and satisfactory precision. Increasing the amount of coating resulted in an increase in maximum binding (B_0; counts obtained with the zero standard), which is desirable, but also in an increase in the nonspecific binding of the tracer and a decrease in the assay sensitivity (at constant antibody dilution). The amount of antibody used in the assay dramatically affected the sensitivity of the calibration curve. Antibody titration curves were performed with both the biotinylated Fab fragments and the monoclonal antibody. The proper dilution was selected so that B_0 decreased to 50% of its value with digoxin concentrations between 1 and 2 μg/L. The standard curves for digoxin, when optimized, are useful for the quantitation of the drug in serum between 0.5 and 5 μg/L. The detection limit of the assay calculated from the point which is two standard deviations of the zero standard below the response of the zero standard is 0.25 μg/L. A typical standard curve using Fab fragments is shown in Figure 2. Calibration curves with the monoclonal antibody-based assay were similar. Working with polyclonal antibodies, we found that the steepness of the calibration curve was greater when we used monovalent Fab fragments instead of the whole antibodies. This observation is similar to the one reported by Hinds et al.[29] for an enzyme-based immunoassay for digoxin.

The precision and recovery of the assay (procedure with Fab fragments) are shown in Tables I and II. The cross-reactivity of the assay (using Fab fragments) is shown in Table III. The specificity of this polyclonal antibody is for the steroid part of the digoxin molecule and thus, compounds having differences only in the sugar part of the molecule, like lanatoside and digoxigenin (not studied), are expected to cross react strongly. Other steroids show negligible cross-reactivity. The specificity of the monoclonal antibody used is reported in detail by Mudgett-Hunter et al.[23] This antibody (affinity constant 4.5×10^{10} M^{-1}) shows specificity for the C_{12}—OH group on the steroid part and secondarily for the sugar moiety of the digoxin molecule. Thus, digitoxin (which lacks the C_{12}—OH group of digoxin) has ~1% cross-reactivity. Digoxigenin, which lacks the sugar part of digoxin, shows ~10% cross-reactivity. Deslanoside and gitoxin show 100 and 3% cross-reactivities, respectively. Other compounds like acetylstrophanthidin, ouabain, and digitoxigenin, and steroids like

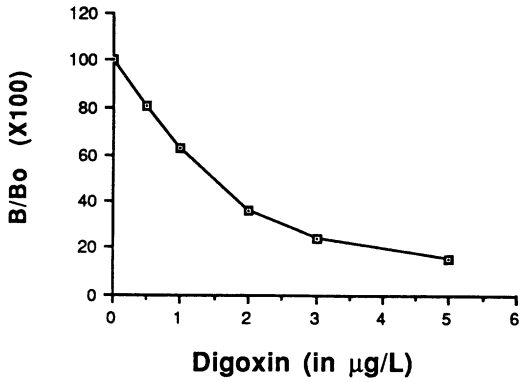

Figure 2—Calibration curve of the proposed assay using biotinylated Fab fragments. A similar curve was obtained with the monoclonal antibody: B_0 is the fluorescence obtained with the zero standard and B the fluorescence of all other standards.

Table I—Precision of the Proposed Digoxin Assay (Fab Fragments Procedure)[a]

Sample	Digoxin, μg/L		
	Mean	SD	%CV
Within-run			
1	0.84	0.07	8.9
2	1.86	0.09	5.0
3	2.95	0.21	7.2
Day-to-Day[b]			
1	0.80	0.09	11.2
2	1.95	0.15	7.7
3	2.90	0.23	7.9

[a] n = 12. [b] Over a period of one month.

Table II—Recovery of Added Digoxin to Serum Samples (Fab Fragments Procedure)

Digoxin, μg/L			
Initially Present	Added	Recovered	% Recovery[a]
0.80	0.90	0.80	89
	1.60	1.90	119
	2.30	2.48	108
0.50	1.60	1.52	94
	2.30	2.22	96
0.80	1.60	1.51	94
	2.30	2.20	96

[a] The mean recovery was 99.4 ± 10.4%.

Table III—Cross-Reactivity of the Proposed Digoxin Assay (Fab Fragments Procedure)

Compound	Percent Cross-Reactivity[a]
Digoxin	100
Lanatoside	64
Digitoxin	6.4
Digitoxigenin	14
Testosterone	<0.001
Prednisone	<0.001
Progesterone	0.01
Spironolactone	<0.001
Cortisol	<0.001
Ouabain	0.01

[a] The cross-reactivity is expressed as the percent ratio of the digoxin equivalent concentration to the cross-reacting substance concentration at the 50% inhibition of maximum binding.

Table IV—Dilution Linearity of the Proposed Digoxin Assay

Sample	Dilution	Expected, μg/L	Observed, μg/L
1	Undil[a]	—	4.6
	1.33	3.5	3.7
	2	2.3	2.7
	4	1.2	1.1
2	Undil	—	3.8
	1.33	2.9	3.0
	2	1.9	2.0
	4	1.0	0.8
3	Undil	—	2.2
	1.33	1.7	1.6
	2	1.1	1.1
	4	0.6	0.6
4	Undil	—	2.6
	1.33	2.0	2.1
	2	1.3	1.6
	4	0.7	0.5

[a] Undiluted.

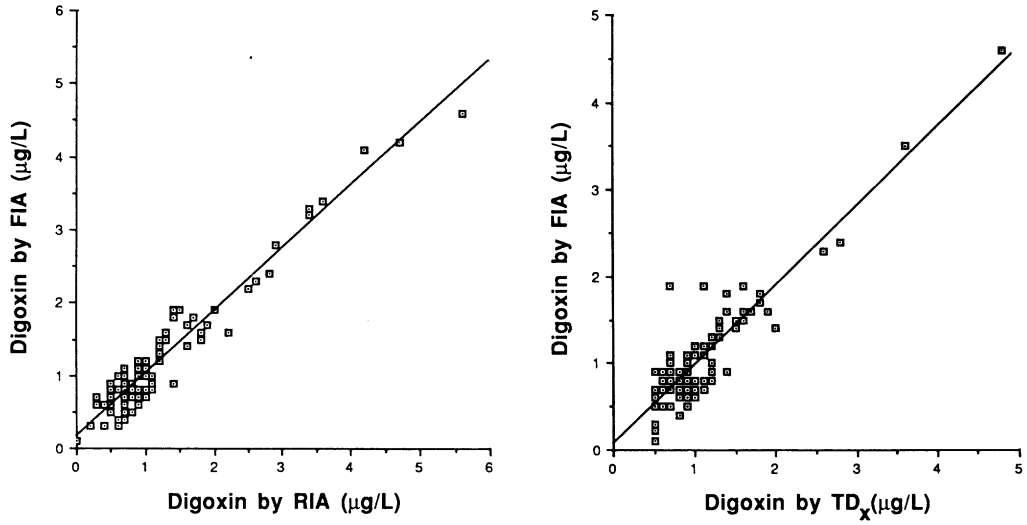

Figure 3—Comparison of the proposed method (Fab fragments) with two widely used procedures (left panel: NEN RIA; right panel: TDx digoxin). The regression equations are: y (proposed) = 0.16 + 0.86x (RIA), r = 0.97 (n = 91); and y (proposed) = 0.07 + 0.91x (TDx), r = 0.92 (n = 85).

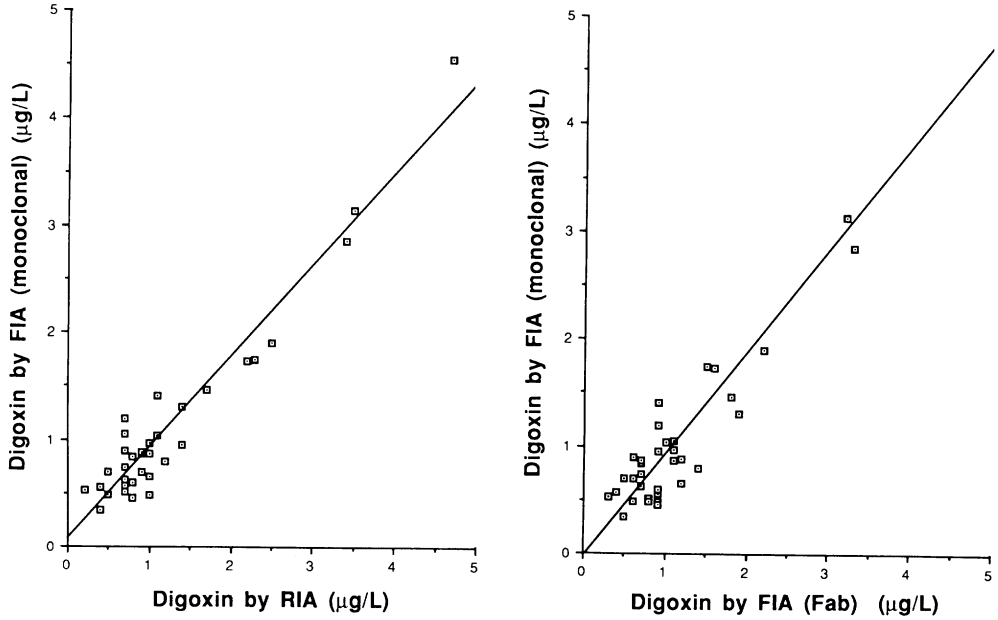

Figure 4—Comparison of the proposed method (monoclonal antibody) with the NEN RIA (left) and the Fab fragments procedure (right). The regression equations are: y (proposed) = 0.07 + 0.85x (RIA), r = 0.96 (n = 35); and y (proposed monoclonal) = −0.03 + 0.96x (proposed Fab fragments), r = 0.94 (n = 35).

cholesterol, testosterone, 17-β-estradiol, progesterone, cortisol, and 4-androstenedione show no significant cross-reactivity.[25]

A number of other high-affinity monoclonal antibodies also prepared by Mudgett-Hunter[23] were studied for their usefulness in immunoassay with the present system. Some gave good dose–response curves (data not shown), but they were not studied further because they showed wide differences in specificity, especially in comparison to the well-established polyclonal antibodies currently used for digoxin quantitation in serum.

Four patients' sera with high digoxin concentrations were diluted with the zero standard and re-assayed (Fab fragments procedure) to study the parallelism of the proposed assay. The results are shown in Table IV. There is good agreement between expected and observed values. To further study the accuracy of the proposed procedure (Fab fragments), we analyzed patient sera with two other well-established methodologies, the TDx digoxin assay and the New England Nuclear RIA procedure. The results are shown in Figure 3. We also analyzed samples with the monoclonal antibody procedure and the comparison results are shown in Figure 4. There is a good agreement between results with all procedures tested.

It has recently been reported that digoxin antibodies used in many commercial kits cross react with unknown substances present in the serum of neonates and pregnant women, amniotic fluid, cord blood serum, placental extracts, and serum of patients with renal or hepatic failure.[30–32] These substances are now known as DLIS (digoxin-like immunoreactive substances). To study the effect of DLIS on the proposed assays, we analyzed 19 cord serum samples by both the Fab and the monoclonal antibody procedure. Values ranged from undetectable to 0.49 μg/L for the monoclonal antibody assay and from undetectable to 0.63 μg/L for the Fab fragments procedure.

The interesting features of the proposed assays can be summarized as follows. The methodology used is new and different from the one used by Helsingius et al.,[25] which is also based on time-resolved fluorescence. In this approach, BCPDA and not Eu^{3+} is used as a label, thus eliminating the problems of Eu^{3+} contamination, as exemplified further in another publication.[9] The assay is nonisotopic and avoids the problems associated with the use and disposal of radioisotopes. The biotin:streptavidin interaction has been incorporated into the system because of a number of advantages; that is, it is a universal detection system, biotinylated antibodies and labeled streptavidin are prepared easily and they are stable reagents, and amplification is introduced. Compared with the automated homogeneous immunoassays for digoxin, the proposed assays are inferior in terms of speed of analysis. However, for analyzing batches of samples, the assay is productive since in ~90 min a whole 96-well plate can be processed. Reading the plates, including data reduction and printing, takes only 5 min on the immunoanalyzer. Apart from the methodology, it is demonstrated here that clinically useful immunoassays can be devised by using high-affinity monoclonal antibodies for digoxin. We anticipate that such antibodies will dominate in commercial immunoassays for the drug during the next 5–10 years.

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