A Unitized Enzyme-Labeled Immunometric Digoxin Assay Suitable for Rapid Testing


An enzyme-labeled immunometric assay has been developed for measuring digoxin concentrations in serum or plasma. Unitized, compartmentalized reagents are used with an automated sample-processing instrument. The enzyme activity of the processed sample, which is directly proportional to the digoxin concentration, is measured by using a reagent strip and the Ames Seralyzer® reflectance photometer. The test takes less than 15 min, and digoxin concentrations are calculated from a two-point calibration line stored in the instrument. Within-run CVs for controls at four concentrations ranged from 2.3% to 3.8%; between-run CVs were from 1.5% to 2.6%. Results obtained with clinical serum samples correlated well (r >0.96) with those obtained by fluorescent polarization immunoassay (Abbott TDx) and RIA (Clinical Assays and NML). This rapid and convenient method for monitoring digoxin concentrations in serum or plasma is particularly well suited for decentralized sites such as emergency rooms, urgent-care centers, and physicians' offices.

Digoxin, a cardiac glycoside derived from the leaf of the plant Digitalis lanata, is used to treat congestive heart failure and atrial fibrillation and to manage atrial flutter and paroxysmal tachycardia (1). Its therapeutic range is generally reported to be 0.8 to 2.0 μg/L in serum. Monitoring digoxin concentrations in serum and observing clinical symptoms can maximize its effectiveness and avoid toxicity (1-5).

Methods used to determine the concentration of digoxin in serum or plasma include radioimmunoassay (6), fluorescence polarization immunoassay (7), fluorescence energy transfer immunoassay (8), cloned enzyme donor immunoassay (9), immunometric assay (10), combined liquid chromatography/radioimmunoassay (11), radial partition immunoassay (12), and a receptor assay (13). We have developed an immunometric assay for digoxin that can be performed in 15 min when used in conjunction with a dry reagent substrate strip analyzed in an Ames "Seralyzer" reflectance photometer.

In this assay (see Figure 1, below) an excess of a monoclonal antibody for digoxin mouse monoclonal antibody binds the digoxin in the sample. The conjugate not bound to digoxin is then removed by treatment with a capture phase: digitoxigenin bound to polyacrylamide beads via an amidopropic acid spacer arm. After separation of the capture phase from the assay solution, the \( \beta \)-D-galactosidase activity of the assay solution is determined by reflectance photometry of a reagent strip containing the substrate dimethylacridinone galactoside (DMAG), to which the sample is applied. This enzyme activity is directly proportional to the digoxin concentration of the sample.

Materials and Methods

Apparatus

Reflectance measurements were made in a Seralyzer reflectance photometer with use of a 630-nm, three-cavity interference filter (Ditric Optics, Hudson, MA). The data generated by the photometer were collected and analyzed by a Model HP-85 computer (Hewlett-Packard, Palo Alto, CA 94304). Absorbance spectra were measured in a Model HP-8450A spectrophotometer (Hewlett-Packard). Before application to reagent strips, samples were processed either by an Ames Sample Processor or a rotator under program control from an Epson HX-40 computer (Epson American, Inc., Torrance, CA 90505).

Materials

The enzyme substrate, DMAG, was prepared according to procedures in U.S. patent 4,810,636 (14). The digitoxigenin-derivatized affinity resin, the capture phase, was prepared according to methods in U.S. patent 4,822,747 (15). The monoclonal antibody was prepared by covalently linking a Fab' fragment of a monoclonal antibody to digoxin and the enzyme label \( \beta \)-D-galactosidase. The mouse antibody, for preparation of the monoclonal antibody, was raised against a bovine serum albumin–digoxigenin conjugate. \( \text{Fab}'(\text{b})_2 \) fragments were prepared by pepsin digestion of Protein A-purified mouse monoclonal antibody. Reduction of the dimer to \( \text{Fab}' \) fragments was done with mercaptoethanol; this treatment generated three sulfhydryls on each \( \text{Fab}' \) fragment. The enzyme, \( \beta \)-D-galactosidase, was activated by first blocking the surface sulfhydryl groups with iodoacetamide, then reacting with the hetero-bifunctional cross-linker succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). Five maleimide groups were incorporated per enzyme molecule by this method. Conjugation of the activated enzyme with a threelfold molar excess of Fab' fragment produced several products, which we separated by preparative electrophoresis. The monoclon conjugate was finally isolated by immunoaffinity chromatography. For further details see European Patent Publication No. 270530 (16).

Reagents

Dry capture phase (30 mg) was dispensed into the right compartment of each vial holder, and the compartment was capped tightly. The liquid conjugate reagent was a 0.4 mmol/L solution of the monoclon conjugate in 15 mmol/L potassium phosphate–25 mmol/L potassium citrate buffer, pH 6.4, containing, per liter, 50 mmol of potassium chloride, 0.4 g of sodium azide, and 1 g of bovine serum albumin. This reagent was dispensed (0.75 mL per vial) into 2-mL glass vials and the vials were capped. The vials containing conjugate were inserted into the left side of the vial holders, next to the compartment containing 30 mg of capture phase.

The reagent paper for measuring \( \beta \)-galactosidase activity was prepared by dipping filter paper into a buffered 15

Miles Inc., Diagnostics Division, Elkhart, IN 46515.
Received June 20, 1989; accepted October 5, 1989.

CLINICAL CHEMISTRY, Vol. 36, No. 2, 1990 201
mmol/L solution of DMAG and drying it in a stream of warm air. The treated paper was then cut into 0.5 × 1 cm sections and mounted with double-sided adhesive on 0.5 × 8.3 cm polystyrene supports. The strips were stored in capped black-plastic bottles with silica gel and molecular sieve desiccant materials.

Calibrators and controls were prepared by mixing gravimetrically prepared digoxin solutions with heat-treated (60 °C for 60 min), filtered normal human serum containing 1 g of sodium azide per liter as preservative.

**Assay Procedure**

The assay procedure is shown in Figure 1. Remove the cap from the conjugate vial, and pipet 30 μL of serum or plasma sample into the conjugate reagent. Remove the cap from the capture-phase compartment, and place the vial holder in the rack of the sample-processing instrument. Secure a hollow connector over the tops of both vials in the vial holder, and press the start button of the sample processor. The sample is now automatically processed for 12 min. During this time, the vial holder is rocked for 2 min to mix the sample with conjugate reagent, incubated statically for 6 min to complete complexation of the conjugate with digoxin, and rotated for 4 min to mix the capture phase with the sample/conjugate solution and thereby remove any free conjugate from the solution. After processing, remove and discard the connector and push a piston filter into the capture-phase compartment, which now contains the assay mixture. The filtrate in the center of the piston filter contains the conjugate, which was bound to the digoxin from the sample.

Place a reagent strip on the Seralyzer reflectance photometer, pipet a 30-μL aliquot of filtrate onto the reagent strip, and push the start key to begin sample analysis. The reflectance is monitored from 40 to 60 s after sample application and converted to a function, L(R), which is linearly proportional to chromophore concentration. The reactivity of the sample is compared with a stored calibration curve, and a calculated digoxin concentration is displayed.

For comparison purposes, we assayed clinical samples containing digoxin by TDx (Abbott Diagnostics, Inc., North Chicago, IL 60064), by Clinical Assays’ RIA (Baxter Travel-nol, Diagnostics, Inc., Cambridge, MA 02139), and by NML™ RIA (Organon Teknika Corporation, Irving, TX 75015), according to the instructions in the respective product inserts.

**Results**

The DMAG Reagent Strip

The spectra of DMAG and its chromophore, dimethylacridin, are shown in Figure 2. Upon hydrolysis of the DMAG, color develops with a peak in the absorption spectrum at 634 nm. We monitored the color formed at 630 nm when conjugate was applied to DMAG reagent strips. Figure 3 shows the kinetic profiles for several conjugate concentrations. When the L(R) rate from 40 to 60 s was plotted vs

---

**Fig. 1.** Assay scheme and sample processing steps for the Seralyzer digoxin assay

**Fig. 2.** The absorbance spectra of 0.19 μmol/L solutions of dimethylacridinone galactoside and dimethylacridinone in sodium borate buffer, 0.1 mol/L, pH 10
We digoxin concentration (bottom to top curve) were 0.0, 0.10, 0.20, 0.30, 0.40, and 0.50 nmol/L.

Conjugate concentration, the relationship was linear up to a concentration of 0.5 nmol/L (Figure 4).

Binding of Digoxin to Conjugate

We determined the time needed for 0.4 nmol/L conjugate to bind the digoxin in a 5 µg/L sample, a concentration that is at the upper end of the assay range. The binding was completed 8 min after the sample was mixed with the conjugate. Samples containing lower concentrations of digoxin were found to bind conjugate more quickly than the 5 µg/L sample. A time of 8 min was, therefore, chosen for the incubation step of the assay.

Binding of Conjugate to Capture Phase

The time courses for the removal of conjugate from solution by various amounts of capture phase were determined (see Figure 5). Both the rate of conjugate removal and the total amount of conjugate removed depended upon the amount of capture phase used. The increased ability of 30 mg over 20 mg of capture phase to remove conjugate from solution is apparent only at times less than 2 min. With both 20 and 30 mg of capture phase, an equilibrium in the capture phase removal is reached in about 2 min. An amount of 30 mg of capture phase and a rotation time of 4 min were chosen for the assay to ensure complete removal of free conjugate.

Criticality of Timing after Sample Processing

In a decentralized laboratory, the operator may not be available to complete the assay immediately after sample processing. Therefore, we examined the criticality of the timing between the completion of sample processing and the insertion of the piston filter. There was no change in the assayed digoxin concentration if the insertion of the piston filter was delayed for up to 60 min after sample processing was completed. We also examined how long the sample could stand after the piston filter was inserted. There was no change in the assayed digoxin concentration when the filtrate was used up to 4 h after the filter was inserted.

Dose Response to Digoxin

The assay for digoxin in serum was performed as shown in Figure 1, and its dose response is shown in Figure 6. The dose response is linear between 0 and 5 µg/L, so a two-point calibration can be used. We chose 0.6 and 3.0 µg/L digoxin for the calibrator concentrations.

Other Assay Variables

Precision and accuracy. These were determined by calibrating the Seralyzer reflectance photometer and assaying several concentrations of serum controls, in triplicate. This was repeated 20 times. Table 1 summarizes the within-run and between-run precision of the assay. The bias of the assay is negligible below 3.0 µg/L, where the therapeutic

We pipetted 780-µL aliquots of serum not containing digoxin, diluted 26-fold in 0.38 nmol/L conjugate solution, onto either 10, 20, or 30 mg of capture phase and mixed them by end-over-end rotation (30 min) for the times shown. After stopping the rotation, we used a piston filter to separate the resin from the assay solution and determined the reactivity of 30 µL of the filtrate. For each capture-phase amount, a serum sample containing 20 µg of digoxin per liter was diluted 26-fold with 0.38 nmol/L conjugate solution, allowed to stand for 10 min, and the reactivity was determined as above, with use of the longest rotation time. The percent background was calculated as (reactivity of the 0 digoxin sample/reactivity of 20 µg/L sample) × 100
range is located, and it becomes slightly negative at digoxin values between 3.0 and 5.0 µg/L. At 4.0 and 5.0 µg/L, the bias was found to be -2.5% and -6.0%, respectively. The precision of the assay was also examined by using clinical serum specimens. Figure 7 is a plot of the results obtained for 99 clinical specimens on two different Seralyzer instruments.

**Sensitivity.** The low-calibrator serum containing 0.60 µg/L of digoxin per liter was mixed with serum not containing digoxin to obtain samples with digoxin concentrations between 0 and 0.40 µg/L. Eighteen replicates each of samples containing 0, 0.10, 0.20, 0.30, and 0.40 µg of digoxin per liter were assayed. The lowest concentration of digoxin that we could distinguish from 0 with 95% confidence was 0.20 µg of digoxin per liter.

**Analytical recovery.** Samples were prepared by mixing equal volumes of clinical samples and digoxin standards, and their digoxin concentrations were determined. For concentrations from 0.8 to 3.4 µg/L, the mean recovery was 98% (range 96–103%).

**System calibration.** The validity of using a stored calibration line was tested by calibrating a Seralyzer and then assaying seven clinical serum specimens at four- to six-day intervals during a month. A freshly thawed aliquot of each serum was used at each time point. The data in Table 2 show that a calibration line can be used for 30 days.

**Cross-reactivity.** We tested the cross-reactivity of some drugs that might be prescribed with digoxin, compounds with chemical structures similar to digoxin, digoxin metabolites, and common over-the-counter medications. At the concentrations in serum noted in parentheses, the following compounds increased the assayed value of a 2.0 µg/L digoxin control by <20%: acetaminophen (6.6 mmol/L), ascorbic acid (5.6 mmol/L), amiodarone (85 µmol/L), caffeine (5.1 mmol/L), carbamazepine (420 µmol/L), cortisone (14 µmol/L), diazepam (350 µmol/L), dihydrodigoxin (64 nmol/L), estradiol (7.4 µmol/L), ethosuximide (3.5 mmol/L), fuphenazine (4.6 µmol/L), ibuprofen (1.3 mmol/L), indomethacin (280 µmol/L), lidocaine (430 µmol/L), meperidine (920 µmol/L), methaqualone (200 µmol/L), methytransferase (490 µmol/L), methyprylon (1.1 mmol/L), N-acetylpromazine (320 µmol/L), ouabain (1.7 µmol/L), phenobarbital (2.2 mmol/L), phenol (530 µmol/L), phenylbutazone (1.6 mmol/L), phenytoin (390 µmol/L), primidone (460 µmol/L), procainamide (3.7 mmol/L), progesterone (1.6 µmol/L), quinidine (310 µmol/L), spironolactone (2.4 µmol/L), testosterone (350 µmol/L), theophylline (5.6 mmol/L), and valproic acid (2.8 mmol/L). Compounds that did increase the assayed concentration of a 2.0 µg/L digoxin control by 20% at the indicated concentrations were morphine (0.64 nmol/L), digoxin (1.05 nmol/L), digoxigenin (1.3 nmol/L), and gitoxin (23 nmol/L).

**Interferences.** Endogenous compounds were examined for interference with the assay. Digoxin was added to samples of serum containing various concentrations of either bilirubin, cholesterol, or triglycerides, which were then assayed. No interference was found up to 150 mg of bilirubin, 3.25 g of cholesterol, or 15 g of triglycerides per liter. To control sera containing digoxin we added either ascorbic acid or uric acid. No interference was seen for the former up to 100 mg/L or for the latter up to 150 mg/L.

Interference from hemoglobin was studied by adding digoxin to hemolyzed plasma and assaying. Non-hemolyzed plasma from the same individual was used as a control. We found a positive interference corresponding to about 1 µg of digoxin per liter for each milligram of hemoglobin per liter; therefore hemolyzed samples must not be used.

To examine the effect of anticoagulants on the assay, we

---

**Table 1. Precision of the Seralyzer Digoxin Assay**

<table>
<thead>
<tr>
<th></th>
<th>0.85</th>
<th>2.00</th>
<th>4.00</th>
<th>5.00</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-run</strong> (n = 60)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, µg/L</td>
<td>0.85</td>
<td>2.00</td>
<td>3.90</td>
<td>4.70</td>
</tr>
<tr>
<td>SD, µg/L</td>
<td>0.03</td>
<td>0.05</td>
<td>0.09</td>
<td>0.18</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.7</td>
<td>2.3</td>
<td>2.3</td>
<td>3.8</td>
</tr>
<tr>
<td><strong>Between-run</strong> (n = 20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, µg/L</td>
<td>0.85</td>
<td>2.00</td>
<td>3.90</td>
<td>4.70</td>
</tr>
<tr>
<td>SD, µg/L</td>
<td>0.01</td>
<td>0.03</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>CV, %</td>
<td>1.5</td>
<td>1.5</td>
<td>2.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>
collected samples of plasma and serum from individuals not taking digoxin and added digoxin to them. Plasma and serum were also sampled from an individual on digoxin therapy. The anticoagulants used were sodium citrate, disodium EDTA, sodium fluoride, lithium heparin, and potassium/ammonium oxalate. None of these anticoagulants interfered with the assay.

Comparison with Other Methods

Clinical serum specimens were assayed by this method and by Abbott’s TDx method (see Figure 8), Clinical Assays’ RIA, and the NML RIA. For comparison with Clinical Assays’ RIA, the results were as follows: $y = 1.03x - 0.07$; $S_{yx} = 0.19 \mu g/L$; $r = 0.96$; $n = 97$. For comparison with the NML RIA, the results were as follows: $y = 0.96x + 0.06$; $S_{yx} = 0.15 \mu g/L$; $r = 0.96$; $n = 91$. Thus this assay correlates well with each of the comparison methods.

Discussion

Dry-reagent immunoassays for monitoring theophylline (17), phenytoin (18), and phenobarbital (19) in serum have previously been described. In the present enzyme-labeled immunometric assay for digoxin in serum, a dry-reagent strip is used in determining the enzyme activity of the conjugate in a processed sample. This activity is proportional to the digoxin concentration in the sample. Only two pipetting steps are used, which minimizes the opportunity for operator error. A sample-processing instrument performs all of the critically timed steps and reagent transfers. Both of the antibody–conjugate binding reactions (i.e., the binding of the digoxin to the conjugate and the binding of the excess conjugate to the capture phase) proceed to relatively stable equilibria; this allows for maximum operator flexibility. Thus the assay can be performed by an operator who has 1 min to set up the sample processing and, after 12 min of processing, has another 2 min within the next hour to complete the assay, using a dry-reagent strip in the Seralyzer reflectance photometer. In a decentralized setting, the 15-min assay time would allow a physician to determine a patient’s serum digoxin concentration while the patient is still at the office.

The linear dose response to digoxin allows use of a two-point calibration that is stable for 30 days. The assay is both precise and accurate, and results correlate well with those obtained by other immunoassays for digoxin.

The assay is not cross-reactive to drugs that might be commonly co-prescribed with digoxin, common over-the-counter medications, naturally occurring hormones, or the digoxin metabolite dihydrodigoxin. The assay is cross-reactive to deslanoside, digitoxin, digoxigenin, and gitoxin, compounds structurally similar to digoxin. However, none of these compounds are co-prescribed with digoxin and therefore should not cause any problem for the use of the assay.

Some serum samples contain endogenous "digoxin-like immunoreactive factors" that cause falsely high digoxin concentrations in certain immunoassays (20). Matheke and Valdes (21) examined the present assay and found very low cross-reactivity with DLIP.

The format for this digoxin immunoassay is particularly appropriate for emergency rooms, urgent-care centers, and physicians' offices, where rapid testing is desirable (22).

References

5. Beller GA, Smith TW, Abelmann WH, Haber E, Hood WB.

Table 2. One-Month Calibration Stability Study

<table>
<thead>
<tr>
<th>Clinical sample</th>
<th>Days after calibration</th>
<th>Sample mean</th>
<th>SD</th>
<th>CV,%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0 4 9 14 18 24 30</td>
<td>1.58</td>
<td>0.04</td>
<td>2.5</td>
</tr>
<tr>
<td>B</td>
<td>1.89 1.89 1.95 2.01 1.99 1.99</td>
<td>1.95</td>
<td>0.05</td>
<td>2.5</td>
</tr>
<tr>
<td>C</td>
<td>3.30 3.38 3.20 3.34 3.48 3.19</td>
<td>3.32</td>
<td>0.10</td>
<td>3.1</td>
</tr>
<tr>
<td>D</td>
<td>0.50 0.57 0.51 0.55 0.52 0.54</td>
<td>0.53</td>
<td>0.03</td>
<td>4.8</td>
</tr>
<tr>
<td>E</td>
<td>1.08 1.04 1.05 1.12 1.01 1.05 1.06</td>
<td>1.06</td>
<td>0.04</td>
<td>3.3</td>
</tr>
<tr>
<td>F</td>
<td>1.44 1.35 1.32 1.39 1.36 1.31 1.38</td>
<td>1.36</td>
<td>0.04</td>
<td>3.2</td>
</tr>
<tr>
<td>G</td>
<td>1.78 1.83 1.77 1.78 1.76 1.64 1.74</td>
<td>1.76</td>
<td>0.06</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Daily mean 1.65 1.65 1.62 1.68 1.67 1.62 1.67 1.65

Fig. 8. Digoxin concentrations measured in clinical serum specimens with the Seralyzer digoxin assay as compared with those obtained by the Abbott TDx digoxin assay

$y = 1.03x - 0.01; S_{yx} = 0.16 \mu g/L; r = 0.96; n = 99$