Digoxin-like Immunoreactivity Eliminated from Serum by Centrifugal Ultrafiltration before Fluorescence Polarization Immunoassay of Digoxin

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Digoxin determined in the Abbott "TDx" by fluorescence polarization immunoassay by the manufacturer's recommended method involving precipitation of protein with 5-sulfosalicylic acid (SSA) is subject to interference from endogenous compounds having digoxin-like immunoreactivity. Guided by the work of Graves et al. (Clin Chem 1986;32:1506–9), we eliminated interference caused by digoxin-like immunoreactivity by substituting ultrafiltration for precipitation with SSA to remove protein. Using the manufacturer's method, we quantified digoxin in serum from 53 patients in three clinically defined groups who were receiving no digoxin, finding apparent digoxin in excess of the 200 ng/L detection limit in 24% of the 17 pregnant women, 59% of the 17 renal-dialysis patients, and all of 19 neonatal cord-blood samples examined. No measurable digoxin immunoreactivity was observed by fluorescence polarization immunoassay for any of the 53 clinically defined patients after removal of protein by ultrafiltration. For 22 men for whom digoxin was prescribed, digoxin measurement after protein removal by SSA and by ultrafiltration correlated well (r = 0.98), with good proportionality (slope = 1.04). Analytical recovery of added digoxin from adulterated serum was 115% after SSA, but 100% after ultrafiltration. Thus, before this assay procedure, we recommend ultrafiltration, to remove digoxin-like interference.

Endogenously produced compounds exhibiting digoxin-like immunoreactivity with antisera for digoxin assays have been described in several clinics defined groups and conditions, including pregnant women (1, 2) neonatal cord-blood (3, 4), patients with renal failure (5), and liver disease (6). These digoxin-like immunoreactive factors (DLIF) are largely protein bound.4 Apparent increases in them observed for patients in renal failure, pregnant women, and newborns are ascribable to changes in protein binding rather than to increased concentrations of digoxin-like factors in serum (7). Graves et al. (8) successfully minimized DLIF interference by removing most serum proteins by ultrafiltration before radioimmunoassay (RIA) of digoxin, RIA being a technique now widely used in the United States for determination of digoxin. However, the College of American Pathologists5 finds that fluorescence polarization immunoassay (FPIA) with the TDx analyzer (Abbott Diagnostics, Dallas, TX 75038) is used for measuring digoxin in 35% of laboratories participating in their survey. This technology is so widely accepted for digoxin measurement because it is based on homogeneous immunoassay and therefore is more easily automated than most other techniques. Analytical matrix problems have been documented previously for FPIA digoxin assay. Porter et al. (9) attributed variability in test results to abnormalities in serum protein concentration, and the manufacturer subsequently substituted 5-sulfosalicylic acid (SSA) for trichloroacetic acid in the protein-precipitation step of their recommended FPIA digoxin assay, apparently in an effort to minimize this serum-protein dependence.

In this study, we examine the susceptibility of FPIA digoxin measurement in the TDx to endogenous DLIF after precipitation of protein with SSA. Also, we compare FPIA digoxin results after SSA precipitation and removal of protein by ultrafiltration in groups of patients for whom endogenous digoxin-like immunoreactivity has been described previously, and in men receiving digoxin therapeutically.

Materials and Methods

Patients' specimens. We quantified digoxin immunoreactivity in serum from 53 clinical subjects: 17 patients on renal dialysis, 17 pregnant women, and cord-blood from 19 neonates. None of these 53 had received digoxin.

In our comparison study, we used leftover serum from specimens submitted to our laboratory for digoxin determination from 22 men for whom digoxin was prescribed.

Protein precipitation. Before digoxin measurement by FPIA, we precipitated serum protein by diluting 200 μL of patient sample with 200 μL of 5-sulfosalicylic acid in equimolar volume of ethanol/water (SSA), in accordance with the manufacturer's recommendations (Abbott Diagnostics, Irving, TX 75038). This mixture is vortex-mixed for 5 s, centrifuged at 9500 × g at room temperature, and digoxin is measured in the resulting supernate. Ultrafiltration of serum. For removal of serum protein by ultrafiltration, we used the "Centrifree Micropartition System" (M, 30 000 cutoff, YM-type membrane; Amicon Corp., Danvers, MA 01923). According to Lukas and de Martino (10), the proportion of digoxin bound to serum protein varies substantially with temperature; approximately 20% of this drug is bound to serum protein at 37 °C, about 4% at 1–4 °C. To optimize digoxin measurement, we equilibrated at 3 °C, 0.5 to 1.0 mL of each serum in separate partition devices before centrifugal ultrafiltration at 2000 × g for 20 min in a refrigerated centrifuge with a 45° fixed-angle rotor.

Fluorescence polarization immunoassay (FPIA). All our digoxin determinations were done in the Abbott "TDx" analyzer with FPIA reagents purchased from Abbott. FPIA digoxin immunoreactivity in patients' samples was extrapolated from fluorescence polarization data stored in the TDx, consisting of analytical digoxin standards purchased from Abbott Diagnostics: 0, 500, 1000, 2000, 3000, and 5000 ng/L. Standard TDx software compensates for twofold dilution of sample with SSA (see above) in reporting digoxin results. Because ultrafiltration involves no specimen dilution, digoxin results from the TDx after ultrafiltration must be divided by two.
Results

We validated the 200 ng/L detection limit for digoxin claimed by the manufacturer for their SSA precipitation and FPIA method, by diluting the assay’s 500 ng/L calibrator to contain 125, 250, and 375 ng of digoxin per liter. For the 375 and 250 ng/L dilutions, we found acceptable digoxin measurements of 410 and 250 ng/L, respectively. For the 125 ng/L dilution, however, we observed a zero digoxin reading. Evidently the detection limit for digoxin is between 125 and 250 ng/L, in accord with the 200 ng/L limit claimed by the manufacturer.

To study analytical recovery, we added precise quantities of crystalline digoxin to plasma (lithium heparin, 14.3 USP units/mL) and serum from an apparently healthy 29-year-old man, who was receiving no digoxin, as diluent for solutions containing 0, 580, 1150, and 2300 ng of the drug per liter. As shown in Table 1, for FPIA measurement in serum, digoxin recoveries were nearly 100% after ultrafiltration and about 115% after precipitation with SSA. Interestingly, serum samples containing no digoxin showed FPIA immunoreactivity corresponding to 130 ng of the drug per liter after SSA precipitation, but 0 ng after ultrafiltration.

We found plasma samples to be unsatisfactory for digoxin measurement after ultrafiltration (data not shown), so only serum was used in this study.

Figure 1 compares digoxin measurement by FPIA after protein removal by SSA precipitation and by ultrafiltration for samples from 22 men who were being treated with digoxin. Regression analysis shows good proportionality between the two protein-removal techniques, as demonstrated by the slope of 1.04 and a correlation coefficient of 0.98. A slight negative bias of digoxin immunoreactivity was seen for ultrafiltration compared with SSA precipitation.

As indicated in Table 2, all 22 samples included in the above proportionality study showed measurable digoxin immunoreactivity after protein precipitation with SSA. For two of these 22 samples, we observed digoxin immunoreactivity that did not exceed the 200 ng/L detection limit defined for the SSA protein-removal technique. For these same two specimens, we observed zero FPIA digoxin immunoreactivity after ultrafiltration.

Table 2 also summarizes our results for the patients. None of these 53 patients had received digoxin, but 48 (91%) nevertheless showed measurable FPIA digoxin immunoreactivity after protein removal with SSA. In contrast, all 53 clinically defined patients showed zero digoxin immunoreactivity after protein removal by ultrafiltration.

After SSA precipitation, four of 17 samples from the pregnant women showed FPIA digoxin immunoreactivity exceeding the assay’s 200 ng/L detection limit, ranging to 600 ng/L in apparent digoxin. For 11 of the other 13 members of this group, we found quantifiable digoxin immunoreactivity but less than the 200 ng/L detection limit for FPIA after SSA precipitation. As indicated in Table 2, all 17 specimens from the pregnant women showed zero FPIA digoxin immunoreactivity after protein removal by ultrafiltration.

Table 2 also shows that serum from 14 of the 17 renal dialysis patients we examined had measurable FPIA digoxin immunoreactivity after protein precipitation with SSA. Immunoreactivity for 10 of the 14 patients showed apparent digoxin in amounts exceeding the assay’s 200 ng/L detection limit, ranging to 640 ng/L. As with the pregnant women, none of the renal-dialysis patients showed any FPIA digoxin immunoreactivity after ultrafiltration.

After protein precipitation with SSA, all 19 of our neonatal cord-blood specimens showed FPIA digoxin immunoreactivity >200 ng/L, ranging from 610 to 1180 ng/L. As with the other groups, none of these 19 neonates demonstrated measurable digoxin immunoreactivity by FPIA after protein removal by ultrafiltration.

Discussion

Our multiple-dilution studies validate the 200 ng/L detection limit claimed by the manufacturer for FPIA digoxin measurement after SSA protein precipitation. Although the 200 ng/L detection limit is sufficient for most digoxin monitoring applications, reliable measurement of lower concentrations may be useful for drug-compliance studies or various pharmacological experiments. Removal of protein by ultrafiltration enhances the FPIA detection limit twofold as compared with SSA precipitation, because ultrafiltration does not require a twofold sample dilution before analysis.

Our studies of digoxin-supplemented samples showed approximately 115% recovery after SSA protein precipitation, 100% after ultrafiltration. Samples containing no digoxin showed digoxin immunoreactivity equal to 130 ng/L after SSA treatment, but the apparent drug quantified was below the 200 ng/L detection limit. Because reporting non-zero numerical results for “drug-free” specimens is misleading, we recommend reporting digoxin as “<200 ng/L” when-

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*Table 1. Analytical Recovery of Digoxin Added to Serum as Measured by FPIA after Removal of Protein by SSA Precipitation or Ultrafiltration

<table>
<thead>
<tr>
<th>Digoxin added, ng/L</th>
<th>SSA precipitation</th>
<th>Ultrafiltration</th>
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<tbody>
<tr>
<td></td>
<td>Digoxin measured,</td>
<td>Recovery, %</td>
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<tr>
<td>2300</td>
<td>2300</td>
<td>114</td>
</tr>
</tbody>
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*Actual digoxin immunoreactivity measured: 130 ng/L

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Fig. 1. FPIA digoxin immunoreactivity after ultrafiltration or SSA precipitation for removal of protein.
ever the FPIA immunoreactivity measured does not exceed the assay's detection limit.

From our correlation study relating digoxin FPIA after protein removal by SSA precipitation and by ultrafiltration, we conclude that there is good proportionality between the two protein-removal methods for the samples from men studied here. The negative bias apparent in Figure 1 was expected because the digoxin recovery observed after ultrafiltration (100%) was proportionately less at all concentrations examined than the recovery observed after SSA precipitation (115%).

Overall, we found that 48 (91%) of the 53 clinically defined patients receiving no digoxin showed quantifiable FPIA digoxin immunoreactivity after protein precipitation with SSA. Twenty-four percent of the pregnant women, 60% of the renal dialysis patients, and all of the neonatal cord-blood specimens we examined demonstrated FPIA digoxin immunoreactivity that exceeded the 200 ng/L detection limit for the commercial method. In contrast, these patients consistently showed zero FPIA digoxin immunoreactivity after protein removal by ultrafiltration.

References