Effect of Protein Concentration on the Determination of Digoxin in Serum by Fluorescence Polarization Immunoassay

William H. Porter, Virginia M. Haver, and Barbara A. Bush

Determination of digoxin by fluorescence polarization immunoassay (FPIA) with the Abbott "TDx" is significantly influenced by the concentration of total serum protein. Each 10 g/L increase in serum protein results in an 8% decrease in measured digoxin. Studies with \(^{3}H\)digoxin confirmed that digoxin binds to the protein pellet during the trichloroacetic acid precipitation step before the immunoassay. Serum protein, or equal concentrations of albumin or \(\gamma\)-globulin, exert an equivalent effect on the apparent digoxin value. Because the total protein concentration of the assay calibrators is low (50 g/L) compared with its reference interval in serum (60–80 g/L), results by FPIA may be expected to be low by an average of 16% (range, 8–24%). Digoxin results by FPIA will be most nearly accurate when the calibrators include a total protein concentration of about 70 g/L. Patients' specimens with abnormally high or low protein content will give falsely high or low results for digoxin.

Additional Keyphrases: albumin \(\cdot \gamma\)-globulin \(\cdot\) calibration \(\cdot\) analytical error \(\cdot\) therapeutic monitoring \(\cdot\) "kit" methods

The recent introduction of a method for determination of serum digoxin by fluorescence polarization immunoassay (FPIA) offers a convenient, rapid, nonisotopic means for monitoring therapy with this cardiac glycoside. Pretreatment of the serum sample with an equal volume of trichloroacetic acid (50 g/L) to precipitate serum proteins before the immunoassay is required to minimize the native background fluorescence produced by the serum proteins, thus resulting in a more favorable signal-to-noise ratio.

Reports comparing digoxin as measured by FPIA with that by various RIA kits have generally been favorable (1–7). The data in some of these studies (2–6), however, suggest that the mean concentrations of digoxin by FPIA generally are lower by 10 to 14% than those by RIA. Moreover, analytical recovery of digoxin, as measured by FPIA, was significantly lower in sera of patients with multiple myeloma than in sera from normal individuals (3). Our own comparison of digoxin measurements by FPIA and RIA indicated that results by FPIA are significantly lower. Examination of the source of this difference led us to conclude that digoxin is bound to the protein precipitate during the pretreatment step, the magnitude of the binding being related to the protein concentration. Thus, differences between the protein concentration in FPIA calibrators (50 g/L) and normal serum (60–80 g/L) largely account for the low results for digoxin by FPIA. As we were completing our studies, a brief report described a similar observation on the effect of sample protein concentration on FPIA results for digoxin (8). However, these authors' interpretation of their findings appears less complete and differs somewhat from ours presented here.

Materials and Methods

Crystalline digoxin was obtained either from United States Pharmacopoeial Convention, Inc., Rockville, MD 20852, or from Sigma Chemical Co., St. Louis, MO 63178. \(^{3}H\)Digoxin was from New England Nuclear, Boston, MA 02118. Human albumin (Cohn Fraction V) and \(\gamma\)-globulin (Cohn Fraction II) were from Sigma. Reagent kits for determination of digoxin by RIA (Quantitone \(^{125}\)I-Digoxin) were purchased from Kallestad Laboratories, Inc., Austin, TX 78701. Reagents for determination of digoxin by FPIA, for use with the "TDx" system, were supplied by Abbott Laboratories, N. Chicago, IL 60064. All other reagents were of analytical-grade.

For the experiments involving \(^{3}H\)digoxin, we first solubilized the protein pellets with Soluene-350, then counted their radioactivity, using Dimilume 30 scintillation fluid, both from Packard Instruments Co., Inc., Downers Grove, IL 60515. Samples were assayed in duplicate; the average counting CV was 6%.
Results

Digoxin concentrations in 65 patients' samples as measured by FPIA and by RIA showed the following correlation (for average of duplicate determinations): FPIA = 0.72 RIA + 0.16, mean FPIA = 1.15 µg/L, mean RIA = 1.36 µg/L, r = 0.9526, S<sub>r</sub>x = 0.19, range FPIA = 0.20–3.66 µg/L, range RIA = 0.05–3.05 µg/L. The low concentrations of digoxin by FPIA were unexpected, because the results obtained for TDM survey samples (Therapeutic Drug Monitoring Program, American Association for Clinical Chemistry) were generally acceptable by either method (Table 1). The mean weighed-in digoxin concentration for 21 TDM survey samples was 1.47 µg/L; the mean concentration measured by RIA and FPIA was 1.55 and 1.48 µg/L, respectively. For either method, the greatest variance from the expected value was 0.2 µg/L.

When either the FPIA kit calibrators or our own prepared calibrators were measured by RIA, the analytical recovery of digoxin was consistently somewhat greater than theoretical (slope = 1.09). On the other hand, when the RIA calibrators or the prepared standards were analyzed by FPIA, the recovery of digoxin was always low (slope < 1.0). Moreover, the analytical recovery of digoxin as measured by FPIA decreased as the total protein concentration in the test samples differed more from the total protein content in the FPIA calibrators. Thus, recovery was lowest (slope = 0.78) when the RIA calibrators (total protein, 70 g/L) were measured by FPIA (calibrator total protein, 50 g/L).

Figure 1 illustrates the effect of protein concentration on digoxin as measured by FPIA, when digoxin concentration is kept constant while protein concentration is varied. Digoxin values equivalent to those expected are obtained when the protein concentration of the sample is near that of the FPIA calibrators (50 g/L). This explains the generally acceptable FPIA results for digoxin in the TDM survey samples, which have a protein concentration similar to that for the FPIA standards (Table 1). As shown in Table 2, for each 10 g/L increase in protein concentration there is approximately an 8% decrease in apparent FPIA-measured digoxin. Moreover, this effect is observed not only for total protein in serum but also, to an equal extent, for pure solutions of albumin or γ-globulin.

The effect of protein concentration on the measurement of digoxin by RIA is less marked than for FPIA and in the opposite direction (Figure 1 and Table 2), the measured digoxin increasing by about 3% for every 10 g/L increase in protein. Unlike the case for FPIA, this protein effect on digoxin measurement by RIA is more closely related to the albumin concentration than to that of γ-globulin (Table 2).

To confirm the suspected loss of digoxin during the pretreatment step with trichloroacetic acid, we performed studies with [3H]digoxin. The partitioning of [3H]digoxin between the supernate and the pellet (Figure 2) confirmed that there is binding of digoxin to the precipitated protein pellet during the pretreatment step.

In an attempt to diminish this binding by protein, we included various organic solvents and detergents in the trichloroacetic acid solution, testing each individually: methanol, ethanol, isopropanol (all 500 mL/L); acetonitrile, 2-methoxyethanol, dimethylsulfoxide (all 250 mL/L); and various concentrations of Triton X-100 surfactant and pentanesulfonic acid. None of these modifications significantly altered the effect of increasing protein concentration on the FPIA result for digoxin. Likewise, substitution of sulfosal-

![Graph showing the relationship between % Recovery of Digoxin and Total Protein (g/L)](image)

**Table 1. Determination of Known Digoxin Concentrations by RIA and FPIA**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein, g/L</th>
<th>Method</th>
<th>m</th>
<th>b</th>
<th>r</th>
<th>n</th>
<th>Range of digoxin concn, µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDM survey samples</td>
<td>54</td>
<td>RIA</td>
<td>1.04</td>
<td>0.03</td>
<td>0.9946</td>
<td>21</td>
<td>0.4–3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FPIA</td>
<td>0.91</td>
<td>0.15</td>
<td>0.9946</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>FPIA calibrators</td>
<td>50</td>
<td>RIA</td>
<td>1.09</td>
<td>0.05</td>
<td>0.9997</td>
<td>5</td>
<td>0.5–5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FPIA</td>
<td>0.78</td>
<td>0.11</td>
<td>0.9999</td>
<td>4</td>
<td>0.5–4.0</td>
</tr>
<tr>
<td>RIA calibrators</td>
<td>70</td>
<td>RIA</td>
<td>1.09</td>
<td>0.08</td>
<td>0.9992</td>
<td>5</td>
<td>1.0–5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FPIA</td>
<td>0.91</td>
<td>0.11</td>
<td>0.9997</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Prepared standards</td>
<td>49</td>
<td>RIA</td>
<td>1.09</td>
<td>0.08</td>
<td>0.9992</td>
<td>5</td>
<td>0.5–5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FPIA</td>
<td>0.86</td>
<td>0.00</td>
<td>0.9996</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

*y = measured value; x = weighed-in concentration.
Prepared standards are digoxin standards prepared in a digoxin-free serum pool.

**Table 2. Effect of Protein Concentration on Digoxin Results**

<table>
<thead>
<tr>
<th>Protein matrix</th>
<th>% change in digoxin concn per 10 g/L increase in protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPIA Serum</td>
<td>-8.0% (-6.5 to -9.5%; n = 3)</td>
</tr>
<tr>
<td>Albumin</td>
<td>-8.0% (-7.0 to -8.5%; n = 5)</td>
</tr>
<tr>
<td>γ-Globulin</td>
<td>-6.0%</td>
</tr>
<tr>
<td>RIA Serum</td>
<td>2.5% (2.0 to 4.5%; n = 3)</td>
</tr>
<tr>
<td>Albumin</td>
<td>3.5% (2.5 to 4.5%; n = 3)</td>
</tr>
<tr>
<td>γ-Globulin</td>
<td>1.0%</td>
</tr>
</tbody>
</table>

*Weighed-in digoxin = 2.0 µg/L. Protein concentration was varied from 15 to 95 g/L. For each determination, the percent change in digoxin concentration was calculated from the slope of the line relating digoxin concentration to protein concentration illustrated in Figure 1.
cyclic acid (30 g/L) for trichloroacetic acid was without beneficial effect.

To compensate for any differences in actual digoxin concentration between calibrators and to adjust the protein concentration of the FPIA standards to be more nearly equal to that for normal serum, we added known concentrations of digoxin to a digoxin-free serum pool having a total protein concentration of 64 g/L. Using these prepared standards to calibrate both the RIA and the FPIA procedures, we measured digoxin in an additional 38 patients' specimens by each method. Under these conditions of assay standardization, there was a much closer overall agreement between methods: FPIA = 0.92 RIA + 0.10, mean FPIA = 1.34 μg/L, mean RIA = 1.35 μg/L, r = 0.9355, S w = 0.28, range FPIA = 0.19–3.90 μg/L, range RIA = 0.06–3.91 μg/L. Indeed, if four specimens identified as outliers (see below) were omitted, the correlation of digoxin values improved to: FPIA = 1.00 RIA – 0.01, mean FPIA and mean RIA = 1.26 μg/L each, r = 0.9768, S w = 0.16, range FPIA = 0.19–3.90 μg/L, range RIA = 0.08–3.91 μg/L.

Discussion

Routine therapeutic monitoring of digoxin has depended on commercial RIA kits, the true accuracy of which remains elusive without the availability of a reference or definitive digoxin method. Because of methodological difficulties, good agreement between various RIA kits for digoxin has not always been realized (9–13). Indeed, consistent differences as great as twofold have been observed between results with some of these kits (11). Variations in the albumin concentration of individual serum samples (9, 14) or of the digoxin standards provided with the kit (15) may account for some of the discrepancies observed. In addition, an endogenous factor with digoxin-like immunoreactivity has been detected in serum from neonates (16–18), patients with renal failure (19), salt-loaded or volume-expanded humans (20) and experimental animals (21), in normal human plasma (22), and in rats with cardiac overload (23). Moreover, the degree of cross reactivity of this interferent varied among different immunoassay kits, with measured values for apparent digoxin as high as 4 μg/L for neonates (16) and 1 μg/L for patients in renal failure (19), none of whom were receiving digoxin therapeutically. For patients with renal impairment who were receiving digoxin therapy, digoxin concentrations measured by three different RIA kits varied by as much as 2.9 μg/L (19). Thus, the existence of this digoxin-like immunoreactive substance(s) and perhaps other as yet unidentified factors, especially in patients with renal failure, further complicates the immunoassay of serum digoxin.

The introduction of nonisotopic immunoassays such as FPIA for the measurement of serum digoxin offers an attractive alternative to RIA with regard to convenience, speed of analysis, and reagent stability. We were therefore disappointed with the initial low FPIA results for digoxin in patients' specimens, as compared with the RIA method we currently use (Kallestad's Quantitope). This finding was particularly disturbing in view of the generally good results obtained for TDM survey specimens, in comparing the expected values for weighed-in digoxin with those measured by both FPIA and RIA.

We eventually related these findings principally to the protein binding of digoxin during the pretreatment step of protein precipitation. Such binding is proportional to total protein concentration over the range of at least 15 to 90 g/L but is independent of protein species. Only when the protein concentration of the sample is near that of the standards, as with the TDM survey samples, will FPIA yield accurate digoxin results. Moreover, these findings explain the low analytical recovery of digoxin in the case of serum from patients with multiple myeloma, as measured by FPIA (3).

Studies on the protein binding of digoxin in serum at normal protein concentrations have clearly established that approximately 25% (18–33%) of the digoxin is bound exclusively to albumin (24, 25). The magnitude of the binding that occurs during the protein-precipitation step is similar to the normal plasma protein binding, but the lack of specificity with regard to the protein fractions involved clearly suggests a different mechanism.

Incidentally, concentrations of serum protein, particularly albumin, also affect the RIA determination of digoxin (9, 14). Because of the binding of digoxin (and of radiolabeled digoxin) to albumin, RIA results for apparent digoxin may vary according to the means of separation of the bound and free fractions (9). Thus, when the albumin remains in solution with antibody, such as with charcoal absorption of free digoxin, there is an apparent increase in the bound fraction of radiolabeled digoxin. On the other hand, when the digoxin–antibody complex is separated from albumin (solid-phase antibody or precipitation by a second antibody) the apparent bound fraction of radiolabeled digoxin decreases. Changes in albumin concentration may therefore introduce errors >25% in the apparent digoxin concentration (9). The Kallestad Quantitope RIA assay we use involves a solid-phase double-antibody complex, so that one would expect increasing concentrations of albumin to result in increasing apparent digoxin values, and this is indeed what is observed (Figure 1).

When both the RIA and FPIA methods were calibrated with the same standards prepared in a digoxin-free serum pool with a total protein concentration similar to that for normal individuals, 34 of 38 patients' samples showed good agreement (<0.3 μg/L). Two of the four discrepant patients' specimens had low concentrations of total serum protein (35 and 41 g/L) relative to that for the prepared calibrators. As
predicted from the data in Table 2, a total difference of about 11% between RIA digoxin and FPIA digoxin would be expected for each 10 g/L difference in protein concentration between the calibrator (64 g/L) and the patients’ samples. For these two patients, such considerations accurately predicted the observed differences in measured digoxin: RIA 1.2/FPIA 1.8 and RIA 2.3/FPIA 3.1 µg/L, respectively. However, for the other two patients, serum protein concentrations (58 and 53 g/L) clearly do not account for the differences in measured digoxin (RIA 0.9/FPIA 0.5 and RIA 4.0/FPIA 2.9 µg/L, respectively); therefore, other factors, perhaps digoxin-like immunoreactive substances, may also be involved.

The patient with the most divergent digoxin values (RIA 4.0/FPIA 2.9 µg/L) was in severe congestive heart failure secondary to mitral valve stenosis, subsequently developed acute renal failure, and died. Although digoxin as measured by either method would be considered to be in the toxic range, the patient experienced no clinical signs of digoxin toxicity. Given a report that interference by the digoxin-like immunoreactive substance in the serum of neonates and uremic patients was generally less for FPIA than for three RIA kits, including a Kallestad kit (26), the FPIA result for this patient may have been more nearly accurate.

We conclude that the immunoassay of serum digoxin clearly is complex. Matrix effects associated with variations in albumin or total protein concentration as well as interference by endogenous digoxin-like factors have been observed. For the most nearly accurate digoxin results by FPIA, standards must be prepared in a matrix with a total protein concentration similar to that of most of the patients, and falsely high or low values may be expected when the protein concentration of the patient’s serum is abnormally low or high. Laboratories using FPIA for routine monitoring of digoxin should be aware of this limitation of the assay. Although Scherrmann and Bourdon (8) suggested that patients’ sera should be diluted twofold with saline before FPIA so that measured values would more closely match those obtained by RIA, we believe that, in view of our data, such an approach would result in a general overestimation of the true digoxin concentration.

References