Improved Liquid Chromatographic/Immunoassay of Digoxin in Serum

Judith A. Stone1,2 and Steven J. Soldin3,4

This HPLC/immunoassay procedure measures digoxin in serum with no interference from digoxin metabolites or digoxin-like factors. We used solid-phase (C18 and Diol) extraction, a C18 column with a tetrahydrofuran/water mobile phase, and final quantification by fluorescence polarization immunoassay. Deslanoside and gitoxigenin were used as the internal standard and the retention-time marker, respectively. The average CV for 300-µL samples at digoxin concentrations between 0.9 and 3.9 nmol/L was 9.3%. Minimum column lifetime with daily use was three months. We also compared results, for 49 samples from patients taking digoxin, obtained with the Abbott "TDx FPIA digoxin I" and the present procedure. Discrepancies between the two methods were substantial for 20% of the samples.

Additional Keyphrases: digoxin-like factors • digoxin metabolism • pediatric chemistry • FPIA compared

The question of accuracy in digoxin immunoassays has come under intense investigation owing to interferences from digoxin metabolites and endogenous digoxin-like factors (DLFs) (1–6). DLFs have been documented in several patient cohorts and the response of different immunoassays to DLFs is varied (7). For purposes of therapeutic drug monitoring, interference in digoxin immunoassays by DLFs in neonates rather than DLFs in patients with renal/hepatic failure or in third-trimester pregnancy are probably of most concern. In contrast, the issue of metabolism is of more interest for the population at large and may be of considerably greater significance. There is evidence for extensive digoxin biotransformation, but there are conflicting reports of the difference between digoxin concentrations as measured in serum by immunoassay or by methods with improved specificity (8–14). Overestimation of digoxin concentrations in serum because of cross-reactivity with metabolites having low bioactivity may contribute to the poor correlation between measured digoxin concentrations in serum and efficacy or toxicity of the drug (15–17). However, the influence of metabolism on the accuracy of routine digoxin analysis is still incompletely defined.

Several research methods are capable of measuring digoxin in serum, i.e., without interference from metabolites or DLFs (8, 11, 18–26). To date, the only assays allowing this type of measurement for serum samples are those involving high-performance liquid-chromatographic (HPLC) separation of the various species, followed by either immunoassay to quantify column eluate fractions (8, 11, 18–21) or post-HPLC-column derivatization (22, 23).

We have developed a modified HPLC/immunoassay to meet our requirements for pediatric pharmacology studies and to serve as a reference procedure in our laboratory. Similar published procedures did not fit our needs because of their requirements for large (1-mL) sample volumes, the short column lifetimes involved, or the co-elution of dihydrodigoxin (DHD) and digoxin. We also compared results for 49 samples from patients taking digoxin, as analyzed with the Abbott TDx fluorescence polarization immunoassay (FPIA) and with our method.

Materials and Methods

Apparatus: The chromatographic apparatus consisted of a Model M6000A solvent-delivery system with a U6K injector (Waters Chromatography Division, Milford, MA). A Model LC-55 UV-variable detector and a Model 203 recorder (both from Perkin-Elmer Corp., Norwalk, CT) were used to monitor and record absorbance. We used a 4.6 × 100 mm column, packed with 3-µm particles of ODS2 (Chromatography Sciences Co., Inc., Montreal, Quebec, Canada) and protected with a precolumn in-line filter (2-µm pore size). For FPIA of digoxin we used the Abbott TDx according to the manufacturer's recommendations ("TDx Digoxin I" immunoassay with trichloroacetic acid as the precipitant). Solid-phase extraction was carried out under reduced pressure with Diol and C18 Sep-Pak cartridges and a Sep-Pak Cartridge Rack (all from Waters Chromatography Division).

Reagents: Tetrahydrofuran and 2-propanol were purchased from Burdick and Jackson, Muskegon, MI; "HPLC"-grade acetonitrile and methanol from Caledon Laboratories Ltd., Georgetown, Ontario, Canada; and "Analytical"-grade ZnSO4 · H2O from BDH Chemicals, Toronto, Ontario, Canada. "Surfasil" siliconizing reagent (primary ingredient, dichlorooctamethyltetrasiloxane) and "BCA Protein Assay" reagent (27) were from Pierce Chemical Co., Rockford, IL.

Glass tubes (12 × 75 mm) used for extraction were siliconized with Surfasil, 100 µL per liter of acetonitrile. Fresh acetonitrile wash solution, 20 mL per liter of water, was made daily. The ZnSO4 solution (100 g/L) was filtered and stored at 4°C; under these conditions it was usable for up to 60 days. Mobile phases were prepared daily by mixing appropriate volumes of organic solvent and distilled, deionized water after filtration through a 0.45-µm (pore size) Durapore filter (Millipore Corp., Milford, MA), then degassing by sonication for 30 min.

Standards: Digoxin and gitoxigenin were from Sigma Chemical Co., St. Louis, MO; digoxin metabolites from Atomergics Chemicals, Farmingdale, NY; and [12C-2H]digoxin from New England Nuclear, Cambridge, MA. Deslanoside was a generous gift from Sandoz Pharmaceuticals, Dorval, Quebec, Canada.

Digoxin and other cardiac glycoside stock standards were dissolved and diluted in ethanol; stored at −20°C, they were...
stable for up to one year. Solutions for preparation of serum standards or for extraction were then diluted in phosphate-buffered (pH 7.4) isotonic saline containing not less than 50 mL of ethanol per liter to prevent adsorption of digoxin to glass, and stored for no longer than one week at 4°C. Aliquots of these serum standards were stored at −20°C for up to one year. We used fresh frozen plasma with digoxin values by FPIA of <0.10 nmol/L, as "digoxin-free serum."

Sample treatment: We extracted the serum samples as follows: Prewash the C18 Sep-Paks with 24 mL of methanol, then with 24 mL of water. Add 25 mL of internal standard (deslanoside, 23.86 nmol/L) and 25 mL of the retention-time marker (gitoxigenin, 28.68 μmol/mL) to 300 mL of a patient's sample, standard, or control. Vortex-mix, pipette 300 μL of this onto the solid phase, and reduce the pressure. Wash the Sep-Paks successively with 1 mL each of water, 100 g/L ZnSO4 solution on ice, and 20 mL/L acetonitrile, then with 3 mL of water. Keep the pressure reduced for several minutes to remove residual water. After manually washing a Diol Sep-Pak with 6 mL of methanol, attach the Diol Sep-Pak below the C18 Sep-Pak (via a disposable plastic pipette tip with 2 cm of the pipet cut off). Elute with 3 mL of methanol through both solid phases, collecting the eluate in siliconized 12 × 75 mm glass test tubes. Dry the eluate under nitrogen at 37°C, reconstitute in 200 μL of mobile phase, vortex-mix for 30 s, then centrifuge at 1100 × g for 15 min before injecting 185 to 195 μL into the HPLC system.

Chromatographic conditions: Monitor the absorbance at 218 nm, with sensitivity set at 0.02 A full-scale. Set the flow rate at 0.6 mL/min with a mobile phase of tetrahydrofuran/water (20.5/79.5 by vol). To prevent carryover, inject 0.2 mL of methanol and 1 mL of tetrahydrofuran after the end of each sample run, then wait 10–15 min before injecting the next sample.

Immunoassay quantification: Start a stopwatch with each injection and collect the entire internal standard (deslanoside eluting at 10–12 min, for example) and digoxin (eluting at 24–30 min, for example) fractions in separate 16 × 100 mm siliconized test tubes. Collect additional 1-min fractions on either side of the digoxin collection time, for a total of four tubes per sample. Dry the eluates under air at 25°C or in a flash evaporator at 40°C. Reconstitute the residue in each tube with 230 μL of digoxin-free serum, vortex-mix for 20 s, centrifuge for 5 min at 1100 × g, and use 200 μL for the FPIA.

We established a calibration curve for the HPLC/immunoassay procedure each week, using 0.6, 1.0, 2.0, 4.0, and 6.0 nmol/L serum standards analyzed as described. We measured digoxin concentration in one low, medium, or high serum control by the method as described daily. A ratio of the FPIA values (digoxin/internal standard) for each standard was used for calibration.

Assay development and clinical studies: For resolution studies to optimize the organic component of the mobile phase, we followed the method of Snyder (28), holding the overall polarity of the mobile phase constant while varying (a) the type of organic component or (b) the ratios of different organic solvents in ternary mobile phases. Interference studies involved direct injection of steroid standards in methanol or analysis by the described method of serum obtained from either specimens of cord blood with FPIA values for digoxin exceeding 0.4 nmol/L or from patients who were taking camenoate or spironolactone. The interference of BD and MD with deslanoside, and DHD with digoxin quantification (see Figure 1), was established by injection of metabolite standards with FPIA reactivity of 2–6 nmol/L, followed by FPIA quantification of the deslanoside and digoxin fractions.

Samples from patients, used for the comparison of methods, were drawn 6–24 h post-dose and were selected randomly from the routine workload at Mt. Sinai Hospital and the Hospital for Sick Children, Toronto, Ontario, Canada. Samples were stored at −20°C from the day of FPIA analysis until the LC/FPIA was performed.

Results

Resolution studies: Maximum resolution for DHD and digoxin was 1.6. Figure 1 shows a chromatogram of metabolite and deslanoside standards; Figure 2, a typical chromatogram of a patient's extracted serum sample. About 45 min is required for chromatography of each extracted sample, including the tetrahydrofuran/methanol wash and subsequent equilibration.

Extraction and quantification: FPIA cross-reactivities were: deslanoside, 150–178% at 0.5–4 nmol/L, and gitoxigenin, 0.1–0.5% at 1–11 μmol/L. The response of the digoxin FPIA to deslanoside was linear from 0.5 to 4.0 nmol/L, the equation for the least squares regression line was y = 1.48x + 0.08 (r = 0.999). The equation for a typical calibration curve (least squares regression) was y = 0.37x + 0.02 (r = 0.999). Minimum column useful lifetime was three months with daily use.

Validation. The imprecision at three concentrations is shown in Table 1. Mean analytical recovery (n = 5) at 1.0, 2.0, and 6.0 nmol/L was 78.8% (SD = 5.7%, CV = 7.2%). Recovery for deslanoside was 78.2% (n = 11, SD = 3.6%; CV = 5%). The assay curve was linear to the limits of the
immunoassay, 6.4 nmol/L. FPIA values for blank fractions (i.e., those without digoxin) were routinely <0.2 nmol/L.

Interference studies: Ten cord-blood samples with FPIA values from 0.47 to 0.79 nmol/L were analyzed by LC/FPIA as described, but without addition of internal standard. All results for deslanoside and digoxin fractions were <0.2 nmol/L. The HPLC profile of immunoreactivity for serum from a month-old infant is shown in Figure 3. FPIA values for the digoxin and deslanoside fractions were <0.2 nmol/L for a patient on 400 mg of intravenously administered candrenate daily (FPIA = 0.7 nmol/L) and a volunteer who took 400 mg of spironolactone daily for three days (FPIA = 0.4 nmol/L). In the described chromatographic system, desmethasone, progesterone, 11α-OH-progesterone, 6-methylprednisolone, and 4-pregnen-3β,20-dione had capacity factors (k') >14.6, with digoxin k' = 11.8 and deslanoside k' = 5.0. Fludrocortisone, prednisone, and cortisone had k' values similar to those for digoxin or deslanoside, and for this reason they were analyzed in blank serum with drug added to give a concentration of 135 μmol/L, but without addition of internal standard. The LC/FPIA results are shown in Table 2. Results of the interference studies with digoxin metabolites for deslanoside and digoxin quantification also appear in Table 2.

Clinical studies: For the 40 samples from adult patients, the FPIA mean was 1.55 nmol/L and the LC/FPIA mean was 1.41 nmol/L. The LC/FPIA mean was 91% of the FPIA mean, and they differed significantly (P <0.01). Table 3 shows the frequency distribution for LC/FPIA values, expressed as a percentage of the FPIA value. The means for all 49 samples (including nine patients from one month to 16 y of age) were FPIA = 1.71 nmol/L and LC/FPIA = 1.46 nmol/L (the mean percentage for LC/FPIA + FPIA was 85%).

Discussion

In developing this assay we sought to combine the excellent recovery of solid-phase extraction with HPLC and FPIA. An absolute requirement was a sample volume ≤0.5 mL, to allow pediatric applications.

Co-elution of DHD and digoxin is commonly reported in the literature (8, 9, 13, 20). Morais et al. (19) described retention of DHD for longer than 24 h on a C₁₈ column with methanol-buffer mobile phase; this is surprising, because almost identical retention times (Rt) for the two compounds have been reported in similar systems. In two partial separations, those by Loo et al. (11) and Kwong and McElraine (23), acetanitrite/water and methanol/ethanol/2-propanol/water were used on C₁₈ columns. The only baseline DHD and digoxin resolution previously published was by Eriksson et al. (26), who used 2-propanol/buffer and a Lichrosorb RP-8 column. However, a second, normal-phase system was used for quantifying the digoxin metabolites digoxigenin, MD, and BD. We observed that separation of DHD and digoxin was influenced by the selectivity (α = R₁/R₂) of both the mobile and C₁₈ stationary phases. Use of the one C₁₈ column (Ultraspere 5 μm, 25 cm, Beckman) investigated offered no improvement over C₁₈. The major difference seen between C₁₈ packings was the extent to which DHD in the chromatogram was split into two peaks, presumably the two DHD epimers identified by Reuning et al. (24). This separation has not previously been reported for a reversed-phase system. The 3-μm ODS2 column we used showed virtually no DHD peak splitting, in contrast to the Perkin-Elmer 3-μm C₁₈ and the Waters 5-μm Novapak. The organic modifier in the mobile phase had a significant effect.

Fig. 2. Chromatogram of a patient's extracted serum sample
The retention-time marker peak (gitoxigenin) is labeled G. Shaded areas: FPIA values for digoxin in column eluate fractions: internal standard at 12-14 min and digoxin at 24-30 min.

Fig. 3. LC/FPIA "chromatogram" of an extracted serum sample from a 30-day-old infant with normal renal function and impaired hepatic function
Digoxin concentrations as measured by FPIA and LC/FPIA methods were 6.7 and 0.2 nmol/L, respectively. The sample was drawn 80 h after a 0.005-mg digoxin dose.
Table 1. Imprecision of the LC/FPIA Method

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within day</td>
<td>0.99</td>
<td>0.09</td>
<td>9.1</td>
</tr>
<tr>
<td>4</td>
<td>1.95</td>
<td>0.15</td>
<td>7.9</td>
</tr>
<tr>
<td>5</td>
<td>6.12</td>
<td>0.32</td>
<td>5.2</td>
</tr>
<tr>
<td>Between day</td>
<td>0.91</td>
<td>0.10</td>
<td>11.0</td>
</tr>
<tr>
<td>11</td>
<td>1.59</td>
<td>0.14</td>
<td>8.8</td>
</tr>
<tr>
<td>10</td>
<td>3.87</td>
<td>0.31</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Table 2. Interference of High Concentrations of Steroids and Digoxin Metabolites in the LC/FPIA

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pre-digoxin</th>
<th>Digoxin</th>
<th>Intern. std.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fludrocortisone*</td>
<td>3.79</td>
<td>1.41</td>
<td>b</td>
</tr>
<tr>
<td>Cortisone*</td>
<td>b</td>
<td>b</td>
<td>1.09</td>
</tr>
<tr>
<td>Prednisone*</td>
<td>1.13</td>
<td>0.42</td>
<td>b</td>
</tr>
<tr>
<td>MD</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>BD</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>DHD</td>
<td>1.21</td>
<td>0.34</td>
<td>b</td>
</tr>
</tbody>
</table>

*Tested at concentrations of 135 μmol/L in serum. **Below the detection limit of the assay. *Tested at 0.5 pmol on column. **Tested at 4.5 pmol on column.

Table 3. Frequency Distribution of Digoxin Concentrations in Patients' Samples as Determined by LC/FPIA

<table>
<thead>
<tr>
<th>No. of</th>
<th>No. samples from</th>
<th>[(LC/FPIA)/FPIA] × 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>samples</td>
<td>adults and pediatric patients</td>
<td></td>
</tr>
<tr>
<td>adults</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4. Effect of Composition of the Mobile Phase on Selectivity for Digoxin (DIG), Metabolites, and Internal Standard (DL)

- **DIG**: digoxin; DL: deslanoside; G, gitoxigenin (other abbreviations in footnote 5).
- **Table 4** shows the influence of 2-propanol as compared with tetrahydrofuran in the mobile phase on the selectivities of internal standard, Rt marker, digoxigenin, MD, and BD. Sensitivity of cardiac glycosides to the different solvent selectivity groups described by Snyder (Group II—2-propanol, Group VII—acetonitrile) (28) has also been discussed by Plum and Daldrup (21). The mobile phase and column described in Methods permitted optimal resolution of the pair most difficult to resolve (DHD/digoxin) and allowed good separation of digoxigenin, MD, deslanoside, and BD (Figure 1). With this system, gitoxigenin is co-eluted with BD, and thus it cannot be used when quantification of this metabolite is desired.

Resolution studies for the cardiac glycosides must be carried out with relatively high (1.0–1.3 nmol) quantities of drug on column, and DHD requires about a 500-fold greater concentration than digoxin for ultraviolet detectability. We found that columns used with nanomole quantities of drug had persistently high background FPIA values, even after extensive washing. We then kept separate columns for resolution studies with nanomole concentrations and for analysis of serum, in which picomole concentrations are present. Syringes, injectors, and tubing were additional sources of contamination after use with relatively high concentrations of digoxin.

In our original extraction procedure we used only a water wash of a C18 Sep-Pak before elution with methanol. We found the useful column lifetime to be only one to two weeks. The ZnSO4 wash of Sep-Paks decreased protein in the methanol eluate by 70%, as compared with the same volume of water wash (protein concentrations were determined after drying and reconstitution in water). The modified extraction procedure prolonged column lifetime to several months, with no significant effect on analytical recovery. Siliconization of the 12 × 75 mm glass tubes is important, because this procedure increases recovery after reconstitution in mobile phase by 15%. We used the addition of ultraviolet-detectable amounts of a cardiac glycoside (gitoxigenin) with low immunoreactivity so that we could collect these fractions with greater accuracy, i.e., a shift in the Rt of the gitoxigenin peak was reflected in the Rt of the internal standard and digoxin fractions. FPIA values >0.2 nmol/L for fractions collected before or after the fraction containing digoxin were added to the digoxin fraction value, if so indicated by a shift in the Rt for gitoxigenin. Samples with FPIA values for digoxin >0.2 nmol/L in these fractions, but *without* a shift in the gitoxigenin Rt, were repeated. When necessary to determine Rt, we injected standards in mobile phase, 25 μL of deslanoside (23.86 nmol/L), 25 μL of gitoxigenin (28.68 μmol/L), and ~1000 counts/min of [3H]digoxin, and collected fractions at 1-min intervals. Using gitoxigenin also allowed us to easily evaluate the performance of the chromatographic system without waiting for FPIA results.

Average imprecision of this rather complex assay is only slightly greater than that of the direct FPIA (CV 9.3% vs 6.7%), and is comparable in the 0.9–2.0 nmol/L range (LC/FPIA; FPIA CV: 11.0% and 10.3%; and 8.8% and 7.6%). Interference in the event of extremely high concentrations of steroids is the only source of known DLF bias. However, the false reactivity of fludrocortisone or prednisone should be detected by high values in the fraction preceding the digoxin Rt. We have used the LC/FPIA assay described to measure digoxin concentrations in the serum of neonates with good success. The method is a useful addition to the spectrum of specific digoxin methods available, because several aspects of digoxin pharmacology in the neonatal population have recently been called into question (7). In the comparison of LC/FPIA and FPIA methods, most (82%) of the LC/FPIA values for adult patients were within 81-100% of the result by FPIA. We arbitrarily considered samples with LC/FPIA values less than 80% of the FPIA value to be significantly discrepant. We divided these discrepant
values into those \(<1.0\ \text{nmol/L}\) and those \(>1.0\ \text{nmol/L}\) and analyzed the distribution by the Chi-square test. The groups did not have significantly different numbers (\(P<0.01\)), indicating that greater imprecision at \(<1.0\ \text{nmol/L}\) did not bias the results.

This study of patients indicates a significant overestimation of serum digoxin concentration as measured by Abbott TDx FPIA in approximately 20% of a general patient population. The TDx Digoxin I FPIA has been shown to have minimal reactivity to DLFs in patients with renal or hepatic failure (29). Therefore the false immunoreactivity we observed presumably is caused by digoxin metabolites in the adult patients and digoxin metabolites and (or) DLFs in the pediatric patients. We saw a much lower incidence of significant discrepancy than was found by Gault et al. (8), who used a specific (HPLC/immunoaesay) digoxin method similar to ours. 18% vs 54% of adult patients with “true digoxin” \(<80%\) of immunoassay. This contrast in incidence of discrepancy may result from methodological differences, i.e., variable reactivity to polar digoxin metabolites by the different antibodies used. A wide range of values for cross-reactivity has been previously reported (8, 10). We conclude that significant overestimation (due to metabolism) of digoxin concentrations in serum during routine therapeutic drug monitoring for the adult and pediatric population does occur, and that the extent of this overestimation may vary greatly, depending on the immunoassay method used.

This work has been supported by the Ontario Heart and Stroke Foundation, Health and Welfare Canada, and the Medical Research Council of Canada.

References