Combined Liquid Chromatography/Radioimmunoassay with Improved Specificity for Serum Digoxin

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This method for assaying digoxin in serum with improved specificity combines small-column extraction of serum, "high-performance" liquid chromatography, and RIA of the eluted fractions. Analytical recoveries of 1.0, 0.5, and 0.1 µg/L standards were 95%, 93%, and 84%, respectively. The CVs for duplicates and replicates of sera with values of 0.5 to 1 µg/L were 4 to 6%. Fifty-nine sera from 50 patients receiving digoxin were so studied. All digoxin metabolites appear to cross react with antibody to digoxin to various degrees. The most polar metabolites were quantitatively the most important, their average cross reactivity being 33%. For eight patients the value for digoxin by the present method was <60% of the RIA value. Sera from nine patients not taking digoxin but with falsely high digoxin values were also studied by the present method. The digoxin peak was well resolved from those for (a) digoxin metabolites (except dihydrodigoxin), (b) digitalis-like factors in neonates and in patients with renal failure or combined hepatic and renal failure, and (c) two cross reacting drugs and their metabolites.

Additional Keyphrases: chromatography, reversed-phase · radioassay compared · cross reactivity · newborns · metabolites present in renal and hepatic failure · "kit" methods · clinical situations in which values are falsely high

Compounds that give falsely high results in radioimmunoassay (RIA) for digoxin reportedly include endogenous compounds in neonates (1, 2) and in pregnancy (3), renal failure (4), and combined hepatic and renal failure (5); drugs and (or) their metabolites (6); and metabolites of digoxin (7–9). More-specific methods are needed, both for clinical and medical–legal purposes.

Most reports indicate that biotransformation of digoxin is of minor significance. However, using a "high-performance" liquid-chromatographic (HPLC)/radioassay method (7) that involved ingestion of [12α-3H]digoxin, we (7, 8) found that some patients, with and without renal failure, had substantial concentrations of digoxin metabolites in their serum (7, 8). Because of the extensive cross reactivity of digoxin metabolites with antibody to digoxin (7, 8) it has been possible to develop an HPLC/RIA method for assaying digoxin and metabolites (7, 9, 10) that avoids the need to administer radioactive digoxin. This method has been substantially modified and simplified. It may be used to assay digoxin alone or, in a longer procedure, to obtain an HPLC elution profile of: (a) digoxin and metabolites, (b) endogenous digitalis-like compounds, and (c) drugs—all of which may interact in commercially available RIAs for digoxin to falsely high values. The method of Loo et al. (11) is somewhat similar to our abbreviated procedure, in which only the HPLC peak for digoxin is determined.

We report here: (a) details of this combined HPLC/RIA method for "true" digoxin and metabolites; (b) its precision and sensitivity; (c) the cross reactivity of certain digoxin metabolites with antibody to digoxin, and (d) our findings of clinically important false concentrations of digoxin caused by some endogenous compounds and certain drugs.

Materials and Methods

Patients

Group Ia: Blood sampled on 40 occasions from a total of 33 patients on long-term therapy with digoxin (longer than one month) was assayed for digoxin by HPLC/RIA and by an RIA (Kallestad, Austin, TX 78701). Blood was collected at steady state for assay, i.e., at least 18 h after the last daily dose. A serum sample from one patient was assayed 10 times for replicate studies; only the average value is included in the results for these 40 sera.

Group Ib: We studied samples from 17 patients on 19 occasions by both HPLC/RIA and the HPLC/radioassay methods, as well as with the Kallestad RIA. Blood for the HPLC/radioassay analysis was usually collected for analysis 6 h after simultaneous ingestion of 150 µCi of radioactive digoxin ([12α-3H]digoxin, cat. no. NET 853-140; New England Nuclear, Boston, MA) and the usual therapeutic dose of unlabeled digoxin. We have shown previously (7) that the proportion of metabolites 6 and 24 h after ingestion of digoxin does not differ greatly; we chose 6 h because, after longer periods, the quantity of radioactivity incorporated in several of the minor metabolites was insufficient for reproducible measurement in patients with normal renal function.

Group II: Nine sera, including one sample of pooled sera from five neonates and two sera from cord bloods, were assayed to ascertain the presence of endogenous digitalis-like factors, or of drugs and (or) their metabolites cross-reacting with antibody to digoxin. None of these patients had received digoxin.

Procedures

Preparation of standards. Digoxin standards were prepared by dissolving 10 mg of digoxin (Boehringer Mann-
We extracted digoxin and its metabolites from 1 mL of serum by using one of three batches of C₁₈ reversed-phase "Bond-Elut" columns (Analytichem, Harbor City, CA 90700), or a single batch of C₁₈ reversed-phase "Sep-Pak" columns (Waters Associates, Milford, MA 01757).

After successively washing the column with 6 mL of methanol and 3 mL of water, we applied 1 mL of serum to the column, washed with 1 mL of water, and discarded the eluate. We then eluted digoxin and metabolites from the column with 3 mL of methanol. After evaporating the eluate in a stream of filtered air at ambient temperature, we reconstituted the residue in 100 μL of 200 mL/L isopropanol, injected it onto a 25 cm × 4.6 mm (i.d.) C₁₈ reversed-phase column (Brownlee, Santa Clara, CA 95050) packed with 5-μm particles, and eluted with a 200 mL/L aqueous solution of isopropanol at a flow rate of 1 mL/min.

For Group Ia samples, we then collected 50 1-mL fractions in RIA tubes for a complete study for 27 sera, but only collected 10 fractions for studies designed simply to define the digoxin peak for 13 sera. The solvent was evaporated under a stream of filtered air, which gives the same results as nitrogen (7), and the residue was reconstituted with 100 μL of digoxin-free plasma for assay by RIA. The HPLC instruments used have been previously reported (7).

We used Kallestad reagents (125I-labeled digoxin) for assay of samples from Groups Ia and Ib, and reagents from New England Nuclear, Boston, MA 02118, for Group II samples. When these kits were used in the usual manner to assay serum digoxin, we have used the abbreviation RIA, in contrast to use of the more-specific present HPLC/RIA method.

Cross-reactivity studies. Studies on the mono- and bis-digitoxosides of digoxigenin, 3α-, 3β-, and 3-keto-digoxigenin, 3β-digoxigenin glucuronide, and dihydrodigoxin involved metabolite concentrations of 5.1 μmol/L (equivalent to 4 μg of digoxin per liter) and use of six kits for digoxin from Kallestad; Clinical Assays, Cambridge, CA 02139; Amersham, Arlington Heights, IL 60005; New England Nuclear; Beckman Instruments, Inc., Fullerton, CA 92634; and Bio-Rad Labs., Richmond, CA 94808.

HPLC/RIA method. We performed this as previously described (7) to assay the 19 sera from patients in Group Ib. In brief: we extracted the samples with Clin-Elut columns, injected the digoxin and metabolites onto a Brownlee C₁₈ reversed-phase column, and eluted with 120 mL/L isopropanol, switching to 200 mL/L at 40 min. Each of 100 chromatographic fractions collected was divided into two aliquots; one was assayed for digoxin and metabolites by use of the Kallestad RIA and the other by counting the tritium radioactivity.

Polar metabolites. The polar metabolites are defined as those appearing as the first two peaks in the HPLC procedure. These compounds are poorly extracted with solvents such as chloroform and methylene chloride. Excluded from the polar metabolites are the mono- and bis-digitoxosides of digoxigenin; 3-keto-, 3α-, and 3β-digoxigenin; and dihydrodigoxin.

To obtain a value for average cross reactivity of the polar group of metabolites with antibody to digoxin, we combined data from the HPLC/RIA and the HPLC/RIA assay. In the latter method we calculated the percentage of digoxin and of each metabolite as the percentage of total radioactivity in plasma represented by each HPLC peak. We calculated the cross reactivity of the polar metabolites from the ratio of the concentration of polar metabolites (in micromoles per liter) as determined by HPLC/RIA and by the combined HPLC/RIA and HPLC/RIA assay methods, as follows:

Cross reactivity of polar metabolites (PM), % =

\[ \frac{[\text{conc } PM \times HPLC/RIA]}{[\text{conc } PM \times HPLC/RIA]} \]

where "calcd conc PM," μmol/L =

\[ \frac{[\text{H}]PM \times HPLC/RIA]}{[\text{H}]PM \times HPLC/RIA} \]

and "true" digoxin concn, μmol/L (by HPLC/RIA)

For example, if the percentage of tritiated polar metabolites as determined by HPLC/RIA is 76% and that of tritiated digoxin is 26%, then for 1.1 μmol/L of digoxin as measured by HPLC/RIA, the calculated concentration of digoxin's polar metabolites will be (76/26) × 1.1 = 3.2 μmol/L. If the concentration of polar metabolites as measured by HPLC/RIA is 0.8 μmol/L, then the cross reactivity of the polar metabolites in this sample is (0.8/3.2) × 100 = 25%.

Results

Validation of HPLC/RIA Method

Mean analytical recovery of digoxin standards added to digoxin-free serum ranged from 84 to 95% (Table 1). All 50 HPLC fractions for 27 Group Ia samples were assayed for "digoxin" (i.e., digoxin and all its metabolites) by RIA. As another measure of recovery, we compared the sum of the results for the 50 fractions with the RIA value for digoxin in an unprocessed serum. The average proportion of digoxin accounted for (as measured by HPLC/RIA) was 97% of that measured by RIA alone (range 88% to 108%, SD 5.3%). For three batches of Bond-Elut columns the recoveries averaged 99% (n = 2), 98% (n = 5), and 94% (n = 11), and recovery was 99% for one batch of Sep-Pak columns (n = 9).

The precision (CV) for 10 replicate determinations for a single serum sample with a mean value for digoxin of 1.30 μg/L by RIA (1.06 μg/L by HPLC/RIA) was 5% (Table 1).

The between-run CV for duplicate determinations by one technician on sera from eight patients was 6% (Table 1); the HPLC/RIA value ranged from 0.3 to 2.3 μg/L (mean 1.2), the maximum difference between duplicates being 0.2 μg/L. We also obtained a between-run CV of 6% on the same duplicates as measured (by different technicians) with the Kallestad RIA.

Retention times for the digoxin peak were quite constant,

**Table 1. Validation of HPLC/RIA Measurements of Digoxin in Serum**

<table>
<thead>
<tr>
<th>A. Assay of weighed digoxin standards in serum</th>
<th>Digoxin, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal value of standard</td>
<td>Measured by HPLC/RIA, mean</td>
</tr>
<tr>
<td>18</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Assay of serum from patients receiving digoxin</th>
<th>Digoxin, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured by RIA, mean</td>
<td>Measured by HPLC/RIA, mean</td>
</tr>
<tr>
<td>10</td>
<td>1.3</td>
</tr>
<tr>
<td>8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

both for a given column during any week (± 1 min) and over the life of a given column (± 2 min).

Figure 1 illustrates the results of the HPLC/RIA method for two digoxin standards in sera and for sera from two patients, one of whom had minimal and the other extensive digoxin biotransformation. The retention time for the digoxin peak was uniformly 34 min. We calculated the digoxin concentration by summing the values for the individual fractions in the digoxin peak.

If we take the retention time of digoxin to be 34 min, the retention time for the bis-digitoxoside is 19 min, for the mono-digitoxoside 12 min, and for α- and β-digitoxigenin and keto-digitoxigenin 9 min (Figure 1A).

Comparison of RIA and HPLC/RIA values. The mean digoxin concentration as determined by RIA in 59 different sera from 50 Group I (a and b) patients was 1.4 μg/L (1.0 μg/L by HPLC/RIA). This difference between the means was significant (p < 0.001 by Student's paired t-test). All HPLC/RIA values for the 59 sera were equal to or less than the corresponding RIA values, the maximum difference being 1.20 μg/L. For eight of the 50 patients the HPLC-RIA value was < 50% of the RIA value (Figure 2, Table 2). Sera from these eight patients and from 24 of the 30 for whom HPLC gave values for digoxin < 50% of the value by the Kallestad RIA were re-assayed by the full HPLC/RIA method (summing all 50 fractions) or the HPLC/radioassay method, or both. For all 32 of these sera the area under all metabolite peaks was roughly proportional to the difference between the RIA and HPLC/RIA methods.

Cross reactivity of polar metabolites. The mean concentrations measured for polar metabolites in the 19 samples assayed by the combined HPLC/RIA and HPLC/radioassay calculation and by the HPLC/RIA method were 0.96 and 0.26 mmol/L, respectively. Excluding two samples in which the concentration of polar metabolites was 0.1 mmol/L or less by the combined method, we determined the average cross reactivity of the polar metabolites with the Kallestad kit antibody to be 33% (SD 25%). The regression line relating the "true" concentration of polar metabolites (combined HPLC/RIA and HPLC/radioassay) and the concentration measured by HPLC/RIA alone was: "true" polar = 4.4 polar (by HPLC/RIA) − 0.2 μmol/L (n = 19, r = 0.88, S_p = 0.79).

Interference from digitalis-like factors and drugs and metabolites (Group II). Figure 3 illustrates HPLC/RIA profiles for four samples containing endogenous components of serum or drugs and metabolites that interact with RIAs for digoxin to give falsely high values. None of the patients shown were receiving digoxin, and none of the elution profiles had a peak with the same retention time as for digoxin.

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**Table 2. Proportion of RIA Value for Serum Digoxin Found by HPLC/RIA to Be "True" Digoxin in 50 Patients**

<table>
<thead>
<tr>
<th>No. patients*</th>
<th>(HPLC/RIA)/RIA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>90–100</td>
</tr>
<tr>
<td>10</td>
<td>80–89</td>
</tr>
<tr>
<td>8</td>
<td>70–79</td>
</tr>
<tr>
<td>12</td>
<td>60–69</td>
</tr>
<tr>
<td>6</td>
<td>50–59</td>
</tr>
<tr>
<td>0</td>
<td>40–49</td>
</tr>
<tr>
<td>2</td>
<td>30–39</td>
</tr>
<tr>
<td>50</td>
<td>Mean 74</td>
</tr>
</tbody>
</table>

*When serum was collected for assays on more than one day (seven patients), the average value was used.

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**Fig. 1.** HPLC/RIA measurement of "true" serum digoxin on a C8 reversed-phase column, eluted with aqueous isopropanol (200 mL/min) at 1 mL/min.

Each 1-mL fraction was assayed for digoxin by RIA and all the values under each digoxin peak were summed. (A) Patient with extensive digoxin metabolism: RIA value for digoxin, 1.9 μg/L; "true" serum digoxin by HPLC/RIA, 0.7 μg/L. Peaks 1 and 5, polar metabolites; 3, digoxigenin; 4, mono-digitoxoside; 5, bis-digitoxoside 6, digoxin. (B) Patient with little digoxin metabolism: RIA digoxin, 1.5 μg/L; "true" serum digoxin (HPLC/RIA), 1.4 μg/L. (C, D) digoxin standards.
Figure 3A shows the profile for a pool of five cord-blood specimens, for which the value for digoxin by HPLC was 0.84 μg/L. The interacting material had a retention time similar to that for the metabolites of digoxin. Serum from a patient with dialysis-dependent renal failure gave a similar elution profile (apparent digoxin = 0.3 μg/L).

Figure 3C shows the multiple peaks obtained with serum from a patient with combined hepatic and renal failure; the peak with a retention time of 25 min contained bile salts in high concentration.

Other cross-reactivity studies. Results of other cross-reactivity studies are listed in Table 3.

Biotransformation in renal failure. Thirty of the 50 patients in Group Ia and Ib had renal failure, mainly dialysis dependent. The mean percentage digoxin in unprocessed serum in the patients with renal failure determined by HPLC/RIA as compared to the concentration assayed by the Kallestad RIA was 71%, not significantly different (p = 0.21) from the value of 77% for those with normal renal function.

Discussion

Assessment of Method

The CV for determinations of duplicates and replicates ranged from 4 to 6%, comparable with CVs reported for unmodified RIAs (12, 13). The low between-day and between-technician CVs for control sera, the high analytical recoveries, and the reproducibility of results with different batches of extraction columns further attest to the validity of this method.

Although it is customary to use an internal standard in this type of chromatographic procedure, to minimize possible errors owing to loss of drug or metabolites because of variations in columns or sample matrix or from pipetting and other technical errors, an HPLC/RIA method for digoxin that does not include an internal standard has been published (11). Finding an appropriate internal standard for this method poses considerable difficulty. A suitable standard would have to have (a) comparable extractability; (b) a retention time differing from that for digoxin, metabolites, for various digitoxis-like factors, and for drugs or their metabolites that react with antibody to digoxin (Figures 1 and 2); and (c) a conveniently short retention time. One possibility is $^{3}H$-dihydrodigoxin, which is eluted with digoxin and cross reacts minimally with the antibody to digoxin. However, this compound is not readily available, and its use would add an additional major step to the procedure and require a liquid-scintillation counter. Commercially available $^{3}H$-digoxin has too low a specific activity and is too reactive with antibody to digoxin to be used.

An alternative to using an internal standard in an assay when only the fractions under the digoxin peak are being assayed is to pool all the fractions outside the digoxin peak; assay an aliquot of the pool for digoxin, add this value to the true digoxin value, and compare the sum to the value for digoxin in the unprocessed serum to calculate recovery. However, this method may miss some nondigoxin-reactive compounds in the procedure. In another study, three of our patients with combined hepatic and renal failure who were not taking digoxin had a concentration of digoxin (by RIA of unprocessed serum) of 0.7, 1.7, and 1.8 μg/L (5). Only about 50% of the material causing the false digoxin values was accounted for in their serum samples, the rest being lost in the Bond-Elut extraction step. If such a patient were receiving digoxin and the losses were thought to be digoxin, a correction based on the "internal" recovery would give a falsely high result for digoxin. In the study reported here, the analytical recoveries obtained by summing RIA digoxin values in all 50 fractions per sample ranged from 88% to 108% (mean 97%); however, none of these patients was jaundiced.

The improved specificity of the method is its major advantage. To date, we have found no drugs, endogenous compounds, or digoxin metabolites (except dihydrodigoxin) that are eluted at the same time as digoxin. Nevertheless, for medical-legal purposes, greater specificity is desirable; with this method we can only say that a compound is not digoxin, but not categorically that it is digoxin.

Major disadvantages are the time involved and the cost. We collect 10 1-mL fractions of the HPLC elute to define the digoxin peak, and determine the digoxin concentration on each fraction by RIA. For duplicate determinations of each of the 10 fractions, the minimum cost per test currently would be about $30, including labor, about 10-fold that estimated for duplicate determinations with the usual RIAs. However, we are investigating the possibility of reducing the number of fractions appreciably without loss of precision.

Digoxin Metabolites

Our values for cross reactivity of the different metabolites as measured with six different kits (Table 3) correspond to those reported elsewhere (7, 9, 14–18).

Biotransformation of digoxin can be extensive (7, 8, 19, 20). In 19 sera from Group 1b studied by HPLC/radioassay, metabolites accounted for from 1 to 99% of the total radioac-
tivity in plasma and averaged 40%, as compared with 35% for the polar metabolites. Thus, the polar metabolites were, on average, 88% of the total metabolites. Of the RIA value for unprocessed serum, 40 to 70% was found to be ascribable to polar metabolites in eight of 50 patients studied by HPLC/RIA (Figure 2). Assay of digoxin by the HPLC/RIA and HPLC/radioassay methods gives equivalent results, because both methods measure the actual amount of digoxin in the serum. However, the HPLC/RIA method gives a lower value than the HPLC/radioassay method for metabolites, because those in the polar group especially have <100% cross reactivity with the antibody to digoxin used in this study. The true metabolite concentration somewhat exceeds twice the difference between the RIA and HPLC/RIA values.

The clinical significance of the polar metabolites relates to the fact that they have an average of 33% cross reactivity with antibody to digoxin, but in all probability they have little cardioactivity (7, 21–24). Thus, a major degree of underdosing could result, as it did in the patient whose results are shown in Figure 1A.

The polar group of metabolites appears to consist of several compounds, including the glucuronide and sulfate conjugates of 3a(epi)-digoxigenin (7, 9, 19). The considerable variation found in apparent cross reactivity (33%, SD 25%) suggests that there may be an important variability in the relative amounts of the individual polar metabolites from patient to patient, and that their cross reactivity may vary.

The less-polar minor metabolites measured by RIA for digoxin probably will have little clinical significance. The mono- and bis-digitoxosides of digoxigenin and 3-keto, 3a-, and 3β-digoxigenin ordinarily are individually <3% and collectively <15% of the sum of digoxin and its metabolites, as found by our HPLC/radioassay (7) of serum and urine, and often <7–8% (7). Also, both the cross reactivity and the cardioactivity of the mono- and bis-digitoxosides of digoxigenin (14–17, 22, 25) are roughly equal to that of digoxin itself. In contrast, the cross reactivity of 3β-digoxigenin in most studies (15–17) approaches 100% but the cardioactivity is only 10 to 50% of that of digoxin.

Dihydroidigoxin, which is co-eluted with digoxin in this method, reportedly (18) had <12% cross reactivity with antibody to digoxin antibody in six commercial digoxin kits. We found values of <3% for six kits and confirmed this 2% value given by Kallestad Labs. For the antibody used in this study. Hence, the contribution of dihydroidigoxin to an RIA value, even when present in maximum reported amounts—e.g., 50% of the total (18, 26)—may be considered trivial. In addition, only about 10% of patients form important amounts of reduced metabolites, and the cardioactivity of dihydroidigoxin and other dihydro metabolites is slight (22, 26). Nevertheless, it is important to appreciate that when a patient is receiving antibiotics that kill the intestinal bacteria that form dihydroidigoxin and dihydro metabolites, there may be a major decrease in dihydroidigoxin and a corresponding increase in digoxin of up to 100%, with the danger of overdose (26).

Endogenous Substances and Drugs Reacting in the Digoxin RIA

Endogenous compounds interacting in the digoxin RIA to give false values (up to 4 μg/L) are in the greatest concentration in serum of neonates (1). Values of varying clinical significance have been found with seven RIA kits (1). Our proposed method, or a simpler modification of it, could well prove of value in the assay of digoxin in neonates, because the peaks for those substances in neonatal sera that interact with antibody to digoxin do not overlap with the digoxin peak (Figure 3A).

Additional situations in which clinically significant false digoxin values have been found by RIA include: (a) pregnancy, with values in the 3rd trimester up to 0.6 μg/L (6); (b) renal failure, with values up to 1 μg/L (6); and (c) combined hepatic and renal failure (Figure 3B), with values up to 1.8 μg/L (5). In all three situations, the values for false digoxin as measured with different kits differed appreciably, possibly related in part to antibody specificity and in part to the RIA methodology. The finding of very high false digoxin RIA values for bile (10 to 30 μg/L) and that 3α-bile salts could account for about 50% of the RIA values (5) suggests that certain endogenously produced compounds may alter the interaction of digoxin and its antibody by nonspecific physicochemical means, such as detergent action.

Drugs—especially steroid derivatives such as spironolactone and its metabolite canrenone, 6-methyl-prednisolone, hydrocortisone, estrogens, progestins, and androgens—cross react with antibody to digoxin (6, 27–29), especially if present in relatively high concentrations. Although their RIA cross reactivity with antibody to digoxin is not often clinically significant, we illustrate that administration of large doses of spironolactone (Figure 3C) and 6-methyl-prednisolone (Figure 3D) can produce clinically important increases in apparent digoxin in the usual RIA, but not with the present HPLC/RIA method.

None of the endogenous digitalis-like factors or drugs we evaluated by HPLC/RIA in this study led to an HPLC/RIA peak with the retention time of digoxin (Figures 1 and 3), but the possibility that such non-digoxin compounds exist can by no means be excluded, and this has medicinal-legal significance. Thus, this method can confirm the absence of digoxin, but can not absolutely prove its presence.

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