Endogenous Digoxin-like Immunoreactive Factors Eliminated from Serum Samples by Hydrophobic Silica-Gel Extraction and Enzyme Immunoassay

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Elimination of endogenous digoxin-like immunoreactive factors (DLIF) that interfere with accurate measurement of digoxin requires use of a highly specific anti-digoxin antibody, or that DLIF be separated from digoxin before immunoassay. Several commercial digoxin-assay kits include a step for separating serum proteins and other substances from digoxin before immunoassay. We tested six different immunoassay methods (some having pretreatment steps) for their ability to detect DLIF in serum from patients in renal failure, pregnant women, and neonates, all of whom were not taking digoxin. Extracting digoxin on a column of derivatized silica-gel eliminated detectable DLIF from serum as measured by enzyme immunoassay (EMIT; Syva Co.), but recovery of added digoxin was quantititative. In contrast, protein precipitation with 5-sulfosalicylic acid left significant amounts of DLIF in samples, most probably because the procedure (TDx assay; Abbott Labs.) disrupted protein-DLIF binding. A glass-bead radioimmunoassay (Imphophase; Corning Medical) had the most digoxin-specific antisera. By preparative silica-gel chromatography of serum we could eliminate or significantly minimize inaccurate digoxin measurements attributable to endogenous DLIF.

Additional Keyphrases: variation, source of - intermethod comparison - "kit" methods

Concentrations of endogenous digoxin-like immunoreactive factors (DLIF), which cross react with antibodies raised against digoxin, are increased in serum from patients with renal failure or hepatic failure, from pregnant women, and from newborn infants (see ref. 1 for review). These factors interfere with accurate measurement of digoxin in such subjects by most immunoassays (2). Substantial evidence suggests that more than 90% of these factors are noncovalently bound to proteins in serum (3). Several commercial digoxin assays include a sample-processing step designed primarily to separate proteins or other serum components from digoxin before digestion is measured by immunoassay. In theory, these processing steps should be useful in eliminating or diminishing the concentration of DLIF in the sample before the antibody-digoxin interaction is measured, with the advantage that antibodies less specific for digoxin could then be used for accurate measurement of the drug (4).

In digoxin immunoassay methods that include such a separation step, if this step is to eliminate DLIF effectively it must not disrupt binding of DLIF to proteins. To test whether current assays meet this condition we selected DLIF-positive serum samples from 20 patients in renal failure, 20 preg-
nant women, and 20 neonates—none of whom were taking digoxin—and measured apparent digoxin (DLIF) in these samples by digoxin immunoassays that did or did not include a pre-analytical separation step. We found that a simple hydrophobic chromatographic separation on derivitized silica-gel eliminated almost all DLIF from the sera of these patients.

Materials and Methods

Selection of patients. We measured DLIF in serum or plasma samples selected to include patients in renal failure (serum creatinine > 25 mg/L), pregnant women in their third trimester, and neonates (one to six days postpartum). None was receiving digoxin. From each of these groups we selected 20 samples with high values for apparent digoxin (DLIF > 0.1 μg of digoxin equivalent per liter), as measured by the "Rainen NEN" digoxin radioimmunoassay (New England Nuclear, North Billerica, MA). Specimens from neonates were pooled as necessary to provide sample volume sufficient for all comparison assays.

Normal healthy subjects not taking digoxin were selected from laboratory personnel and their sera were verified not to contain measurable DLIF by the NEN assay. We also selected sera from 15 adult patients with normal renal function (serum creatinine < 15 mg/L) who were taking digoxin and used these samples for the assay-comparison study.

Digoxin assays. We analyzed serum or heparinized-plasma samples within four weeks of blood drawing (samples were stored at −20 °C until analysis). Table 1 lists the digoxin immunoassays we used, each according to the manufacturer's instructions.

The only assay not available commercially at the time of this study was the EMIT C-B digoxin assay (Syva Co., Palo Alto, CA), designed for use with the Cobas-Bio (C-B) centrifugal analyzer (Roche Diagnostics, Nutley, NJ). This assay contained anti-digoxin antibodies raised in rabbit, digoxin labeled with glucose-6-phosphate dehydrogenase (EC 1.1.1.49), hydrophobic bonded-phase silica gel in 0.5 × 0.8 cm (1-mL volume) disposable extraction columns (Bond

<table>
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<th>Table 1. Assays Used for Measuring Digoxin and DLIF</th>
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<td>Digoxin assay</td>
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<tr>
<td>Rainen digoxin (NEN)</td>
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SSA is 5-sulfosalicylic acid.
Elut, manufactured for Syva by Analyticchem International, Harbor City, CA, and glucose 6-phosphate substrate in buffer.

We loaded 500 μL of sample, calibrators, or controls onto the columns and washed each column with 1.0 mL of 0.1 mol/L HCl, followed by 1.0 mL of de-ionized water. Labeled test tubes were placed under each column, and the fraction to be analyzed for digoxin was eluted with 0.35 mL of methanol/water (60/40, by vol). This extraction procedure takes approximately 15 min for 20 samples. All washes and elutions were speeded by use of reduced pressure. These extracts were then analyzed by the routine EMIT procedure in the Cobas-Bio as indicated by Syva.

**Analytical recovery of digoxin and DLIF.** We added various amounts of digoxin (Sigma Chemical Co., St. Louis, MO) dissolved in the methanol/water mixture to aliquots of a pool of digoxin-free serum (as determined by the NEN assay) obtained from healthy subjects, then assayed these samples with the Syva, NEN, and Gammacoat (Clinical Assays, Cambridge, MA) kits. Analytical recovery of DLIF (in digoxin equivalents) measured by these three assay kits was determined by adding increasing concentrations of bovine adrenal extracts to digoxin-free serum pools. To determine the effect of DLIF on analytical recovery of digoxin, we added known amounts of digoxin to DLIF-positive (by NEN) serum samples from each of the three patient groups and re-assayed these samples by the EMIT C-B assay. The lowest concentration of digoxin detectable by these methods (sensitivity) was 0.15 (Gammacoat), 0.02 (Immophase), 0.05 (TDx), 0.04 (aca), and 0.07 (EMIT C-B) μg/L, calculated by adding two standard deviations of the response of 10 replicates of a digoxin-free serum to the standard-curve zero values.

Details of preparation of these DLIF extracts will be described elsewhere (manuscript in preparation) but, in brief, bovine adrenals were homogenized, sonicated, and diluted with ammonium acetate buffer (10 mmol/L, pH 8.0) to 2 mL per gram of tissue, then centrifuged. The supernate was purified by chromatography on G-25 Sephadex, the post salt fraction (3) being collected, lyophilized, and resuspended in water.

Fig. 1. DLIF measured as “apparent digoxin” in (A) 20 pooled samples collected from infants, ages one to six days; (B) samples collected from 20 third-trimester pregnant women; (C) samples collected from 20 patients with renal failure (values for serum creatinine >25 mg/L); and (D) samples collected from 10 healthy laboratory personnel

None was taking digoxin. The data points enclosed by a square or circle are results for those two samples having the highest DLIF values by EMIT C-B.
Results

**DLIF in serum from neonates.** We compared DLIF (apparent digoxin) concentrations measured in the same 20 sample pools from neonates by five of the digoxin assays listed in Table 1. Most assays detected substantial concentrations of DLIF in this group of patients (Figure 1A). The greatest concentrations were measured by the Gammacoat and TDx assays, which indicated that all the neonate sample pools contained at least 0.35 μg of digoxin equivalent per liter, with values as high as 1.4 μg/L being measured. Immophase and the **acon** detected >0.1 μg/L in all samples except two that were measured as zero by the **acon**. In contrast, the column extraction/EMIT C-B assay detected very little DLIF, three-fourths of the samples giving values <0.1 μg/L and half of them zero. The two samples with the most DLIF by EMIT C-B also gave high results for the other assays.

**Pregnancy.** For this group of subjects, the EMIT C-B assay pretreatment step completely eliminated any detectable DLIF. The Immophase and the **acon** measured all samples as <0.2 μg/L, whereas the other two assays measured substantial DLIF concentrations (Figure 1B).

**Renal failure.** The EMIT C-B assay detected no DLIF in 15 of the 20 samples from patients in renal failure; in the other five samples, DLIF was <0.2 μg/L (Figure 1C). The Immophase assay measured significantly lower DLIF values than did the other methods in these samples, as it also had done for samples from pregnant women. The samples with the two highest values measured by EMIT C-B gave considerably higher results when assayed by the other methods.

**Normal controls.** Apparent digoxin results in serum from 10 healthy control individuals are also shown in Figure 1. With both the Gammacoat and the **acon** methods, concentrations of apparent digoxin were measurable, those by Gammacoat being the higher.

**Analytical recovery of digoxin and DLIF.** To assess recovery of digoxin, we added digoxin to digoxin-free serum and analyzed these samples by the EMIT C-B assay. The results (Figure 2) demonstrate quantitative recovery of digoxin by that assay. We also tested recovery of digoxin eluted from the hydrophobic columns by loading serum samples containing tritiated digoxin (New England Nuclear) onto the columns and then collecting and counting radioactivity in the individual wash steps. Ninety-nine percent of the tritiated digoxin was eluted in the methanol wash step, the same fraction in which digoxin is measured by the EMIT C-B assay.

We tested the EMIT C-B assay for recovery of digoxin in the presence of DLIF by adding digoxin to serum from individual samples from the patient groups that previously had tested positive for DLIF. The presence of DLIF had no effect on the measured digoxin in serum from renal-disease patients or pregnant women (Table 2). However, some DLIF was also detected along with the added digoxin in the specimens from neonates.

We also tested the recovery of DLIF (extracted from bovine adrenals) by the EMIT, NEN, and Gammacoat assays by adding DLIF extracts to digoxin-free and to DLIF-free serum and then measuring these samples by the same three assays. Figure 3 shows that the EMIT C-B assay consistently gave the lowest values for DLIF. Comparison of digoxin values for 15 adults (without renal failure) taking digoxin by the Immophase and EMIT C-B assays gave linear-regression correlation parameters of: Immophase = 0.986 (EMIT C-B) - 0.14, r = 0.985, p <0.001, SE = 0.107 μg/L (data not shown).

**Discussion**

The EMIT C-B enzyme immunoassay involves a hydrophobic column preassay separation-step; this eliminated or

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**Table 2. Analytical Recovery of Digoxin by Combined Hydrophobic Chromatography/EMIT Assay in the Presence of Endogenous DLIF**

| Sample            | [A] Conc, μg/L | [B] Added digoxin measured by Syva | [C - A]/[B]: “Apparent” recovery of digoxin, % |
|-------------------|----------------|----------------------------------|--------------------------------|----------------|
| DUF-free pool     | 0.08 0         | 0.90                             | 0.91                            | 101             |
| Renal patient 1   | 0.41 0         | 0.90                             | 0.87                            | 96              |
| Renal patient 2   | 0.36 0         | 0.90                             | 0.90                            | 100             |
| Neonate 1         | 1.24 0.49      | 0.90                             | 1.25                            | 85              |
| Neonate 2         | 1.00 0.19      | 0.90                             | 1.05                            | 96              |
| Pregnancy 1       | 0.41 0.04      | 0.90                             | 0.91                            | 97              |
| Pregnancy 2       | 0.33 0.05      | 0.90                             | 1.04                            | 110             |

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**Fig. 2.** Analytical recovery of digoxin added to digoxin-free serum. Mean of duplicate measurements are shown. Solid line, line of identity (100% recovery), for comparison.

**Fig. 3.** Detection of DUF from bovine adrenal extracts added to and diluted with digoxin-free and DUF-free serum.
considerably decreased the incidence of false-positive digoxin results attributable to endogenous DLIF in our three patient groups. To consistently eliminate detection of DLIF, a digoxin immunoassay would have to have an antibody sufficiently specific for digoxin not to interact with DLIF or, alternatively, would have to include a procedure for separating DLIF from digoxin before assay. Most DLIF is bound to proteins in serum (3, 5). If the hypothesis that DLIF is primarily measured by immunoassay only when it is not bound or "loosely-bound" to proteins (1) is valid, then it follows that, to work effectively, the separation step must minimally disrupt the protein–DLIF interaction.

In this study we compared five methodologically distinct digoxin immunoassays for their ability to detect DLIF. The Gammacoat and Immophase digoxin assays involve straightforward radioimmunoassays, with no sample-pre-treatment or separation steps—i.e., these two assays rely exclusively on the specificity of the antibodies. In the aca a ouabain-affinity column is used that separates free antibody from the digoxin-bound antibody–enzyme complex before analysis. That separation approach, however, would not be expected to eliminate detection of DLIF because the DLIF interacts with the anti-digoxin antibody before the ouabain-mediated column separation. The TDX assay includes a protein-precipitation step with 5-sulfosalicylic acid before assay of the protein-free supernates. The EMIT C-B assay includes a hydrophobic column pretreatment of serum, designed to remove proteins and other potentially interfering substances from the subsequent immunoassay. Our results demonstrate that two of these assays (EMIT C-B and Immophase) either do not detect or considerably decrease detection of DLIF in serum from patients known to have relatively high concentrations of these factors.

Endogenous DLIFs are low-molecular-mass (200–1000 Da), water-soluble, neutral molecules not possessing carboxylic or primary amine groups (3). More than 90% of DLIF is tightly but reversibly bound to proteins in serum (3, 5). The protein-bound DLIF, not readily liberated from the protein by heating, is removable from protein by dialysis (3). In patients with renal failure, as well as in third-trimester pregnant women and neonates, there is an apparent increase of the more "loosely-bound" DLIF component such that it becomes more readily accessible to the antibody—and therefore detectable by conventional immunoassays (2). This gives rise to a high incidence of false-positive results for digoxin in these particular patient groups. The pre-assay hydrophobic chromatographic procedure described here effectively separates protein-bound DLIF from digoxin, thereby providing DLIF-free samples for subsequent analysis. This gentle approach for protein separation apparently does not disrupt the protein–DLIF interaction as observed with harsher protein-precipitation procedures such as that used the TDX assay. Indeed, detection of DLIF by the TDX assay similar to that reported here has been reported by others (6).

In addition to eliminating detection of DLIF in human serum, this new hydrophobic-chromatography/EMIT technique demonstrated excellent recovery of added digoxin and significantly decreased detection of DLIF isolated from bovine adrenals. Recovery of digoxin in vivo was comparable to that for a well-established radioimmunoassay, as evidenced by the excellent linear regression obtained on comparing Immophase with EMIT C-B. Evidently, hydrophobic chromatography does extract both unbound and protein-bound digoxin from the serum sample.

Approaches have been proposed for minimizing interference of DLIF in digoxin measurements such as ultrafiltration (7), altering immunoassay incubation times (7, 8), and "high-performance" liquid chromatography (9). Advantages of the EMIT C-B method include its simple and rapid performance, availability from a commercial source, and that it has now been characterized for the detection of DLIF in three patient groups in which DLIF has been described. The results of this study also confirm our earlier hypothesis (1) that binding of DLIF to serum proteins plays an important role in the detection of DLIF by most presently available immunoassays used for measuring digoxin.

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