Digoxin-like Immunoreactivity in Serum from Neonates and Infants Reduced by Centrifugal Ultrafiltration and Fluorescence Polarization Immunoassay

John E. Ray,1 Domnita Crisan,2 and Denise L. Howrie3

We evaluated the TDx Digoxin II (Abbott) modified procedure for interference from digoxin-like immunoreactive factors (DLIF) in pediatric patients. The effectiveness of centrifugal ultrafiltration as a means of removing DLIF interference from the serum of such patients was assessed. We used sera from 40 patients who had not received digoxin, whom we divided into two age groups: 30 neonates (<34 days postpartum) and 10 infants (younger than six months). Digoxin-like immunoreactivity was detected in 34 of 41 (83%) neonatal specimens (range 0.2–1.0 µg/L) and 16 of 25 (60%) infants’ specimens (range 0.2–1.3 µg/L). Centrifugal ultrafiltration of serum specimens from these patients reduced but did not eliminate the DLIF interference in some specimens. A comparison of concentrations of DLIF in serum with various other patients’ characteristics demonstrated a strong correlation (r = 0.915; P = 0.0001) between DLIF and serum bilirubin in the infants. Apparent digoxin concentrations from 19 serum and serum ultrafiltrate samples collected from 13 patients (four neonates and nine infants) who were treated with digoxin showed a good correlation (r = 0.97); however, the serum samples showed a positive bias of 0.39 µg/L. We conclude that the TDx Digoxin II modified procedure is still subject to considerable DLIF interference in these two pediatric populations. This interference can be reduced in some serum specimens, but cannot be eliminated completely as others reported.

Additional Keyphrases: bilirubin · pediatric chemistry · variation, source of

The presence of digoxin-like immunoreactive factors (DLIF) makes interpretation of digoxin concentrations in the serum difficult. DLIF have been detected in blood samples from patients with renal or liver dysfunction, from pregnant women, and from neonates, as analyzed by the Abbott TDx Digoxin II procedure (1–5). The procedure, modified recently to utilize a more specific antibody, is claimed to have reduced interference from DLIF. However, only limited data evaluating DLIF interference in the TDx Digoxin II modified procedure are available (6, 7).

Various methods have been described to eliminate the interference caused by digoxin-like factors in immunoassay techniques (4, 8–11). Christenson et al. (4) used centrifugal ultrafiltration to produce filtrates free of DLIF. Serum samples from renal dialysis patients, pregnant women, and neonatal cord blood were ultrafiltered, and the DLIF in the filtrate was quantified by fluorescence polarization immunoassay (TDx Digoxin II procedure).

Several investigators have attempted to find a correlation between the concentrations of DLIF in serum or urine and various patients’ characteristics (12–16). Phelps et al. (13) reported that patients in a DLIF-positive group were younger than those in the DLIF-negative group and that a greater percentage of infants younger than six months had measurable DLIF. However, they were unable to correlate DLIF concentration with age. Time-course studies in full-term newborn infants suggest that concentrations of DLIF in serum are greatest at birth (16). However, studies in premature neonates suggest that DLIF in serum increase during the first few postnatal days, then decrease (12, 15). Wolach et al. (16) demonstrated a significant correlation between serum concentrations of DLIF and bilirubin in 23 jaundiced newborns between three and five days postpartum. Further, Clerico et al. (14) examined urine samples from newborns and reported significant correlations between concentrations of DLIF and concentrations of electrolytes and creatinine.

The detection of apparent digoxin concentrations in the sera of patients not receiving digoxin is further complicated by the fact that certain drugs and metabolites cross-react with the antibodies used in immunoassays to quantify digoxin. The cross-reactivity of spironolactone and its metabolites has been evaluated in some immunoassays, including fluorescence polarization immunoassay (17). This interference is increased when spironolactone or its metabolites accumulate in patients with renal and hepatic impairment (18). Other drugs (i.e., hydrocortisone and epinephrine) that do not cross-react in the immunoassays produce considerable digoxin immunoreactivity after intravenous administration to sheep or dogs in doses commonly used for humans (19).

In the present study we examined the degree of DLIF interference in the TDx Digoxin II modified procedure in neonates and in infants younger than six months. We evaluated centrifugal ultrafiltration as a means of removing DLIF interference from pediatric specimens and also investigated the relationship of DLIF in serum with various patients’ characteristics (including the effects of spironolactone or steroid administration) in an attempt to identify pediatric patients who might demonstrate marked DLIF interference.

1 Department of Clinical Pharmacology & Toxicology, St. Vincent’s Hospital, Sydney, Australia. Departments of 2 Pathology and 3 Pharmacy Practice, University of Pittsburgh, PA 15213.

Received June 1, 1990; accepted November 2, 1990.
Patients' specimens: Digoxin-like immunoreactivity was determined in 41 serum specimens taken from 30 neonates and in 25 serum specimens from 10 infants who had not received digoxin therapy. The age (postpartum) and weight (mean ± SD) of the neonates were 7.6 ± 7.6 days and 2.9 ± 0.9 kg, respectively; the infants were 110.8 ± 27.1 days old and weighed 5.5 ± 1.2 kg. Results obtained with the TDx Digoxin II modified technique (Abbott Diagnostics Div. Australia, Sydney, NSW) before and after centrifugal ultrafiltration were compared for 19 serum specimens collected from 13 patients treated with digoxin (four neonates and nine infants). Blood samples were not collected specifically for this study but were taken from specimens arriving in the department for routine investigation. Samples were pooled over a 12-h period for a patient when single samples had insufficient volume for complete analysis.

Digoxin methods: In the TDx Digoxin II method, a 30 g/L solution of sulfosalicylic acid in water/methanol (1/1 by vol) is used for protein precipitation, instead of the trichloroacetic acid used in TDx Digoxin I method. The only other modification is in the "pretreatment solution," in which the phosphate buffer has been changed to Tris. Greater antibody specificity has been claimed for the TDx Digoxin II modified kit; however, the procedure is identical to the TDx Digoxin II method (Abbott Diagnostics Technical Communication).

Centrifugal ultrafiltration: We followed the method of Christenson et al. (4), equilibrating 1.0-mL serum samples at 4°C in separate partition devices (Centrifree Micropartition System; Amicon Corp., Danvers, MA 01923) before subjecting them to centrifugal ultrafiltration at 2000 × g for 20 min in a refrigerated centrifuge. The proportion of digoxin bound to serum proteins is ~4% at 4°C (4). We mixed the filtrate (250 µL) to ensure homogeneity and analyzed it with the TDx Digoxin II modified procedure. Unlike the manufacturer's protocol, the filtrates were not diluted with 5-sulfosalicylic acid reagent; therefore, the digoxin results from the TDx, after ultrafiltration, were divided by two to correct for the standard TDx software compensation.

DLIF detection: We used the detection limit for digoxin claimed by the manufacturer for the TDx Digoxin II modified method (0.2 µg/L) to indicate the presence of DLIF in neonates and infants not receiving digoxin. We quantified the DLIF concentrations in the serum and ultrafiltrate samples, then calculated the concentrations of DLIF in the serum retentate (i.e., the unfiltered serum remaining in the sample reservoir after centrifugal ultrafiltration) as the difference.

Statistical analysis: Mann–Whitney U and unpaired Student's t-test were used to evaluate the significance between concentrations of DLIF and various patients' characteristics such as sex, prematurity, etc. Multiple regression analysis and correlation were used to compare the concentrations of DLIF in serum with age, weight, concentrations of bilirubin, creatinine, and blood urea nitrogen. Data are reported as mean ± SD, and significance was defined as P < 0.05. We tested data for a gaussian distribution by using the Shapiro and Wilk test with D'Agostino's D statistic (20).

Results

Forty-one specimens from neonates and 25 specimens from infants who had not received digoxin were analyzed for DLIF interference. Digoxin-like immunoreactivity was detected in 34 (83%) of the neonatal specimens, with concentrations ranging between 0.2 and 1.0 µg/L (mean 0.43 µg/L) (Figure 1). Similarly, DLIF were detected in 16 (60%) of the infants' specimens, with a range of 0.2–1.3 µg/L and a mean of 0.56 µg/L. Other characteristics of the patients whose serum concentrations of DLIF exceeded 0.2 µg/L are shown in Table 1.

Centrifugal ultrafiltration of the serum specimens and subsequent analysis of the ultrafiltrates by the TDx Digoxin II modified procedure decreased the DLIF interference in most specimens (Figure 1). In neonates, DLIF were completely removed from 22 (65%) of the serum specimens (Figure 1, dark bars). However, DLIF were detected in 12 (35%) of the ultrafiltrates (range: 0.2–0.6 µg/L); in five of these samples, all of the DLIF detected in serum were recovered in the ultrafiltrate. Similar
Table 1. Characteristics (Mean ± SD) of Patients Not Receiving Digoxin but with Detectable DLIF in Their Serum

<table>
<thead>
<tr>
<th></th>
<th>Neonates</th>
<th>Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>20/14</td>
<td>3/13</td>
</tr>
<tr>
<td>Age, days postpartum</td>
<td>7.6 ± 7.6</td>
<td>110.8 ± 27.1</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>2.9 ± 0.9</td>
<td>5.5 ± 1.2</td>
</tr>
<tr>
<td>Premature, yes/no</td>
<td>10/24</td>
<td>2/13*</td>
</tr>
<tr>
<td>Bilirubin, mg/L</td>
<td>43 ± 34</td>
<td>93 ± 92</td>
</tr>
<tr>
<td>(74 ± 58)b</td>
<td>(158 ± 157)b</td>
<td></td>
</tr>
<tr>
<td>Creatinine, mg/L</td>
<td>8.6 ± 7.6</td>
<td>3.5 ± 2.4</td>
</tr>
<tr>
<td>(75 ± 67)b</td>
<td>(31 ± 12)b</td>
<td></td>
</tr>
<tr>
<td>Urea N, mg/L</td>
<td>209 ± 120</td>
<td>247 ± 282</td>
</tr>
<tr>
<td>(7.5 ± 4.3)c</td>
<td>(8.8 ± 10.0)c</td>
<td></td>
</tr>
</tbody>
</table>

*Not known in one infant. b μmol/L in parentheses. c mmol/L in parentheses.

Results were seen in infants: DLIF were removed from 13 (81%) of the serum specimens, and three (19%) ultrafiltrate specimens had detectable DLIF (0.5 μg/L each).

We saw no demonstrable relationship between mean concentrations of DLIF in serum and the age, weight, sex, or prematurity of the neonates and infants. Similarly, no correlation could be demonstrated between serum creatinine or blood urea nitrogen and concentrations of DLIF in serum, ultrafiltrate, and serum retentate. However, the concentration of DLIF in serum retentate decreased with increasing age in neonates ($r = 0.525; P = 0.009$), and we observed a strong correlation in infants for concentrations of DLIF in serum ($r = 0.915; P = 0.0001$) and concentrations of DLIF in ultrafiltrate ($r = 0.853; P = 0.0001$) compared with bilirubin concentrations in serum. A significant but weaker correlation was observed between concentrations of DLIF in serum retentate and bilirubin in serum ($r = 0.694; P = 0.003$) in the same population. Only one serum sample from the infant group had a concentration of bilirubin great enough to interfere with the fluorescence polarization immunoassay; the correlation between concentrations of DLIF and bilirubin in serum remained significant when this result was eliminated from the analysis ($r = 0.918; P = 0.0005$). A relationship between serum bilirubin and concentrations of DLIF in serum, ultrafiltrate, or serum retentate was not apparent in neonates.

Although aldactone had been prescribed to six neonates who had detectable DLIF in serum (DLIF range 0.2–0.6 μg/L), there was no significant difference between the mean concentrations of DLIF in serum in this group and the mean concentrations of DLIF in serum from untreated neonates ($P = 0.10$). (Aldactone was not given to any of the infants.) Similarly, there was no difference between mean (±SD) concentrations of DLIF in the serum of neonates or infants who had received steroids (three neonates: 0.30 ± 0.20 μg/L; 12 infants: 0.44 ± 0.36 μg/L) and those who had not (38 neonates: 0.38 ± 0.23 μg/L; 13 infants: 0.32 ± 0.38 μg/L).

Results of digoxin analysis in serum by the TDx Digoxin II modified procedure ($y$) were compared with those by the same procedure for centrifugal ultrafiltrates ($x$) of specimens from 13 patients receiving digoxin. The results were strongly correlated ($r = 0.97$; $y = 0.94 x + 0.39 μg/L$; slope not significantly different from 1.0); however, analysis of the serum samples demonstrated a positive bias of 0.39 μg/L. Further, in three specimens, the contribution of DLIF to the total digoxin concentration was >0.5 μg/L.

Discussion

The TDx Digoxin II modified procedure appears to have reduced interference from DLIF in adult patients with renal or hepatic disease (6, 7). However, the DLIF interference may be important in patients with combined hepatic and renal dysfunction when samples from these patients are analyzed by the TDx Digoxin II procedure (6). Centrifugal ultrafiltration appears to be effective and economical in removing DLIF from the serum of these adult patients (4, 6).

We were unable to demonstrate similar reductions in interference from DLIF in pediatric patients. We found that centrifugal ultrafiltration of serum from neonates and infants did not produce filtrates free of DLIF as previously reported (4). Centrifugal ultrafiltration of serum from pediatric patients did partially remove DLIF from filtrates, indicating a multicomponent nature, or the presence of protein-bound fractions of DLIF, or both (21). Further, our data suggest that (a) the nature and (or) protein-binding of DLIF may vary between individuals; (b) the nature of DLIF in adults may differ from that of pediatric patients (7); and (c) neonatal cord blood, which has been used to evaluate the cross-reactivity of DLIF in various immunoassays (presumably to enable the collection of adequate volumes for analysis), may not be an appropriate means of estimating digoxin-like immunoreactivity in serum taken directly from neonates and infants.

Evaluating the extent of interference from DLIF in the serum of patients receiving digoxin has been difficult. We compared apparent digoxin concentrations in the serum and ultrafiltrate samples taken from pediatric patients receiving digoxin. Although centrifugal ultrafiltration reduced the interference from DLIF in some pediatric specimens, the procedure may produce misleading results. Therefore, we recommend caution when interpreting digoxin concentrations in ultrafiltrates produced by centrifugal ultrafiltration from pediatric patients receiving digoxin.

Several attempts have been made to correlate various patients' characteristics with concentrations of DLIF in the serum of neonates or infants. Some found that concentrations of DLIF in preterm and term neonates decrease with increasing age (12, 15, 16), whereas others could not demonstrate a correlation between DLIF concentrations and age (13). No correlation between gender and concentration of DLIF in the serum of neonates or infants has been demonstrated (12, 13, 15), and reports of a relationship between concentrations of DLIF and birth weight are conflicting (12, 16).
We were unable to demonstrate a relationship between mean concentrations of DLIF in serum and the age, weight, gender, or prematurity of the neonates and infants. The conflicting reports regarding a relationship between concentrations of DLIF in serum and the age or weight of neonates and infants may result partly from the different methods used to quantify DLIF. Moreover, interference from DLIF was evaluated in cord blood in some studies, whereas serum from specimens collected for routine diagnostic analysis was used in others. Comparison of data generated from cord blood samples and serum collected directly from pediatric patients may not be appropriate.

Any relationship between DLIF and age is further complicated by the fact that we were able to demonstrate a significant decrease in concentrations of DLIF in serum retentate (the serum remaining after ultrafiltration) with increasing age of neonates. Perhaps more than one digoxin-like immunoreactive factor exists; if the concentration–time profiles of these factors vary in vivo or if their affinity for the antibodies used in the immunoassays differs, then accurate determination of a relationship with age, weight, etc. will be possible only when the contribution of each factor can be evaluated independently.

Although Phelps et al. (13) reported a weakly positive correlation between concentrations of DLIF and creatinine in serum, they were unable to demonstrate a relationship between concentrations of DLIF and creatinine >6 mg/L (53 µmol/L). We could not demonstrate a relationship between serum creatinine concentrations and the concentration of DLIF in serum, ultrafiltrate, or serum retentate. Similar results were obtained for blood urea nitrogen.

Bilirubin has a strong affinity for serum albumin (22), is not ultrafiltered (23), and does not interfere with the TDx Digoxin II modified procedure at concentrations of total bilirubin <200 mg/L (343 µmol/L) (24). The strong correlation we observed between concentrations of DLIF in serum and serum bilirubin suggests a relationship between hyperbilirubinemia in infants and serum concentrations of DLIF, as proposed by Wolach et al. (16). They argue that DLIF might decrease bilirubin clearance by impeding bile flow and excretion through inhibiting either glucuronyl transferase or Na,K ATPase-modulated bile flow (or both).

Although spironolactone, steroids, and (or) their metabolites are known to interfere with some immunoassays (17–19), we were unable to demonstrate any effect from the administration of these compounds to the pediatric patients we studied. Further study in a larger population may be necessary to resolve the issue.

We conclude that the TDx Digoxin II modified procedure is still subject to considerable interference from DLIF in the two pediatric populations studied. This interference can be reduced in some serum specimens by centrifugal ultrafiltration but cannot be eliminated completely as previously reported. Further, we recom-

We thank Darla Lower for her skillful technical assistance. J. E. R. was supported by a scholarship from Abbott Diagnostics Division Australia.

References
Displacement of Phenytoin from Serum Protein Carriers by Antibiotics: Studies with Ceftriaxone, Nafcillin, and Sulframethoxazole

Amitava Dasgupta,1 David A. Dennen,1 Roger Dean,2 and Ronald W. McLawhon1

Increased concentrations of free phenytoin in serum, attributable to the displacement of this anticonvulsant by other drugs, e.g., valproic acid and salicylic acid, have been reported. We observed in vitro and in vivo displacement of phenytoin by the antibiotics ceftriaxone, nafcillin, and sulframethoxazole. In vitro studies demonstrated statistically significant (P < 0.05) increases in free phenytoin after the addition of specific antibiotics to patients' sera and to phenytoin-supplemented sera from controls. Concentrations of free phenytoin in vivo, predicted by an equation we have found to be accurate for albumin concentrations ≥32 g/L, were consistently underestimated in patients receiving concomitant therapy with the antibiotics studied. The concentrations of free phenytoin decreased towards the predicted values when the antibiotic therapy was discontinued. We conclude that ceftriaxone, nafcillin, and sulframethoxazole can displace phenytoin from the usual protein carriers found in serum, in vitro and in vivo.

Additional Keyphrases: variation, source of anticonvulsant drugs, fluorescence polarization immunoassay

Phenytoin, an anticonvulsant widely used in the management of seizure disorders, is highly protein-bound (90%) in serum. Displacement of phenytoin by other strongly protein-bound drugs such as valproic acid and salicylic acid at steady-state has been reported (1-4). Such displacements of phenytoin from protein-binding sites increase the free fraction of phenytoin while decreasing the total concentration of phenytoin in serum of patients receiving the drug at a constant dosage (5). Because free phenytoin represents the pharmacologically active fraction of the drug and has a very narrow therapeutic range, 4–8 μmol/L, such drug–drug interactions may increase the concentrations of free phenytoin and lead to inappropriate phenytoin dosing if doses are based solely on total phenytoin concentrations in serum.

Nafcillin and ceftriaxone, β-lactam-type antibiotics commonly used to prevent and treat infections of the central nervous system, are frequently administered concurrently with phenytoin. Ceftriaxone is 84–93% bound in serum and exhibits concentration-dependent albumin binding over the usual therapeutic range (6, 7). Nafcillin is 87% bound to protein in the serum (8). Bactrim ("Septra") contains trimethoprim and sulfamethoxazole, 1:5 (by wt), with sulfamethoxazole 65% bound to protein, and trimethoprim only 35% protein bound (9, 10). We present the first report on in vivo and in vitro displacement of phenytoin by ceftriaxone, nafcillin, and sulframethoxazole.

Materials and Methods

Ceftriaxone was purchased from Hoffmann-La Roche Pharmaceuticals, Inc., Nutley, NJ; nafcillin from Wyeth Laboratories, Inc., Philadelphia, PA; and sulfamethoxazole from Sigma Chemical Co., St. Louis, MO.

For in vitro experiments, sera from patients receiving phenytoin and no other concurrent medications that could potentially displace phenytoin were combined to create two serum pools with albumin concentrations of 25 and 32 g/L. A third serum pool with albumin at 45 g/L was prepared from sera of apparently healthy volunteers and subsequently supplemented to achieve a phenytoin concentration of 66.1 μmol/L. Microliter quantities of ceftriaxone or nafcillin were added to 1-mL aliquots from the three pools to mimic peak therapeutic concentrations of each drug in serum. Samples were incubated at 37 °C for 12 h in a water bath. Concentrations of free phenytoin were measured in the protein-free ultrafiltrates (prepared with the Micropartition System from Amicon, Danvers, MA, by centrifuging at 1162 × g at 25 °C) by a fluorescence polarization immunoassay (Abbott Laboratories, Abbott Park, IL). Total