Variability among Commercially Available Digoxin Radioimmunoassay Kits in Cross Reactivity to Dihydroadigoxin

William G. Kramer, Nikki L. Kinnear, and H. Keith Morgan

We evaluated four commercially available $^{125}$I-digoxin radioimmunoassay kits with regard to their ability to cross react with the digoxin metabolite dihydroadigoxin. We prepared dihydroadigoxin serum samples in digoxin-free serum over the concentration range 0.4 to 5.0 µg/liter and assayed them with each kit according to the manufacturer's instructions. The metabolite was able to displace the $^{125}$I-labeled digoxin derivative from the antibody supplied with all four kits. However, the extent of the cross reactivity depended on the kit, ranging from essentially zero to a high degree of interference. Dihydroadigoxin is the only metabolite of digoxin to have been quantitated in human serum, and may comprise up to 30% of total glycosides. Over the clinical and therapeutic range of serum digoxin concentrations, enough dihydroadigoxin can be produced to interfere in the determination of serum digoxin concentrations by this method. We suggest that laboratories evaluate their specific kit with regard to cross reactivity to this metabolite.

Radioimmunoassay is a rapid and sensitive method for measuring digoxin in serum or plasma. A question exists, however, regarding the specificity of the method with respect to the metabolites of digoxin. Cross reactivity between a digoxin antibody and the primary metabolites digoxigenin-bis-digitoxoside and digoxigenin-mono-digitoxoside was demonstrated by Stoll et al. (1). Kramer et al. (2) reported a lack of specificity with respect to the relatively cardio-inactive metabolite dihydroadigoxin (3) for the digoxin antibody provided with a commercially available kit. This cross reactivity is significant, because dihydroadigoxin is the only metabolite found in measurable quantities (0.18-0.36 µg/liter) in plasma (4). Although most digoxin antibodies are produced by methods analogous to that originally described by Smith et al. (5), differences among commercially available kits could alter their susceptibility to this metabolite. These differences include different antibody titer, as suggested by different concentration ranges, different methods for the separation of bound and free antigen, and different methods of radiolabeling either digoxin or a digoxin derivative. Consequently, there is a potential variability among commercially available kits with respect to cross-reactivity with dihydroadigoxin. This paper reports the results of an investigation of this variability.

Materials and Methods

The following digoxin radioimmunoassay kits were either purchased or obtained by donation: Lanoxitext $\gamma$ Digoxin Radio-immunoassay Kit with $^{125}$I Digoxin, Wellcome Reagents Limited, Beckenham, England; IMMO PHASE Digoxin ($^{125}$I) Radioimmunoassay Test System, Corning Medical Diagnostics, Medfield, Mass.; Digoxin IMMUTOPE Kit, E. R. Squibb & Sons, Inc., Princeton, N.J.; and RIA-Mat Digoxin I 125 Kit, Mallinckrodt Nuclear, St. Louis, Mo. Although in each kit $^{125}$I was the radiolabel, the method of coupling the isotope to either digoxin or a digoxin derivative varied. The effective concentration range varied among the kits, and in each a different method of separating bound from free antigen is used.

Dihydroadigoxin in plasma was prepared in digoxin-free plasma in concentrations of 0.4, 1.0, 2.0, 3.0, and 5.0 µg/liter with dihydroadigoxin from Bio-Dynamics/bmc, Indianapolis, Ind. The purity of the commercially-obtained dihydroadigoxin with respect to digoxin contamination was established by high-resolution nuclear magnetic resonance (Bruker HX90 Spectrometer). The signal in the digoxin spectrum at $\delta$ 5.65 due to the olefinic proton was absent in the dihydroadigoxin spectrum, indicating that any unsaturated impurity was less than 1%. The presence of all three sugar moieties in the dihydroadigoxin molecule was confirmed by elemental analysis. Samples were then assayed with each kit according to the instructions provided by the kit manufacturer. For each kit, four assay runs were performed, with each sample and standard run in duplicate, yielding eight values for each sample and standard for each kit. Measurement of radioactivity was performed in a calibrated Automatic Gamma-Well System, Model 1195, Searle Analytical, Inc.

Results

For each kit, the percent of $^{125}$I-labeled digoxin or digoxin derivative bound to the antibody was calculated for each digoxin and dihydroadigoxin sample; these data are plotted in Figure 1 as a function of sample concentration. For purposes of calculating the apparent digoxin concentration for each dihydroadigoxin sample from each kit, the calibration curves were linearized by plotting the ratio of the percent bound at zero concentration to the percent bound at a given concentration against concentration (7). The slopes and intercepts of the calibration curves were calculated by least-squares regression. For all four kits, the coefficient of variation of the slope and intercept of the standard curve (averaged for the four runs) was less than 10%. The apparent digoxin concentrations for the dihydroadigoxin samples were calculated from the regression equations, and are presented in Table 1.

---

Department of Pharmaceutics and Institute for Cardiovascular Studies, College of Pharmacy, University of Houston, Houston, Tex. 77004.

Received July 5, 1977; accepted Oct. 14, 1977.
Table 1. Apparent Digoxin Concentrations Produced by Dihydropyridoxin for Four Kits (A-D)

<table>
<thead>
<tr>
<th>Dihydropyridoxin concn, µg/liter</th>
<th>Apparent digoxin concn, µg/liter</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>(0.09)</td>
<td>0.027</td>
<td>0.036</td>
<td>0.072</td>
<td>0.27</td>
</tr>
<tr>
<td>1.0</td>
<td>(0.057)</td>
<td>0.051</td>
<td>0.042</td>
<td>0.042</td>
<td>0.38</td>
</tr>
<tr>
<td>2.0</td>
<td>(0.083)</td>
<td>0.087</td>
<td>0.13</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>(0.084)</td>
<td>0.11</td>
<td>0.25</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>(0.086)</td>
<td>0.18</td>
<td>0.42</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

* Due to the small difference in molecular weights (2 mass units), the concentrations of both compounds are expressed in the more clinically significant units rather than in molar units.

* Each value is the mean of four runs. Numbers in parentheses is the SD.

Fig. 1. Displacement of 125I-labeled digoxin derivative from the antibody by digoxin (●) and dihydrodigoxin (▲). Each point is the mean of eight values (four runs, two samples/run). The SD's were too small to be shown on the same scale.

Please note that in the figure, table, and discussion the kits are referred to by the letters A through D. The order of presentation of the data does not correspond to the order in which the kits were listed above.

Discussion

It is apparent from examination of Figure 1 that dihydrodigoxin will displace the radiolabeled antigen from the antibody supplied with all four kits. Except for some dihydrodigoxin concentrations of <1 µg/liter for some of the kits, the percentage of bound 125I-labeled digoxin derivative was significantly decreased by the metabolite as compared to the percent bound at zero unlabeled antigen concentration (unpaired Student's t-test, P < .05 or lower). More importantly, the degree of displacement by the metabolite relative to that of digoxin varied among the kits.

Although each kit displayed a statistically significant displacement of the 125I-labeled digoxin derivative by dihydrodigoxin, it is more relevant to examine cross reactivity from the standpoint of clinical significance—i.e., if the degree of interference could become important at therapeutically observed concentrations of digoxin or dihydrodigoxin, or both. As can be seen from the data in Table 1, a given concentration of dihydrodigoxin yields an apparent digoxin concentration which depends on which kit is used. For example, an 0.4 µg/liter sample gives an apparent concentration of 0.027 µg/liter with Kit A, essentially below detectable limits, but a 10-fold greater apparent concentration, 0.27 µg/liter, with Kit D. This concentration is now well within the limits of the procedure, particularly if digoxin itself is present to further increase the displacement of labeled derivative from the antibody. Kit A was the least sensitive to the metabolite, showing almost no cross reactivity up to a dihydrodigoxin concentration of 3.0 µg/liter, well above usually observed digoxin concentrations in serum. Kits B and C were about equal with respect to cross reactivity, and were somewhat more sensitive to the metabolite than was Kit A. Kit D appeared to be the most sensitive, yielding measurable apparent values even at the 0.4 µg/liter concentration. The results obtained with Kit D agree reasonably with those obtained by Kramer et al. (2), who used the kit manufactured by Schwarz/Mann. Consequently, it may be concluded that not only will dihydrodigoxin interfere with the digoxin radioimmunoassay, but the degree of interference depends on the kit that is being used.

Although dihydrodigoxin is not the only digoxin metabolite that will interfere in the digoxin radioimmunoassay, it is the only one yet found in measurable concentrations in some plasma samples (4). It would appear that this digoxin metabolite may be present in plasma and also the degree of metabolism may be patient-dependent (5), ranging up to 30% of total glycosides. Consequently, with therapeutic serum dihydrodigoxin concentrations from 0.4 to 2.0 µg/liter and clinically observed therapeutic and toxic concentrations spanning an even greater range, many patients quite probably produce enough dihydrodigoxin to give falsely high values for serum digoxin as determined by radioimmunoassay. Coupled with the dependence of cross reactivity on the source of the kit, this adds considerable question as to the validity of this method for the determination of serum digoxin concentrations unless the particular kit being used has been evaluated and found not to cross-react with dihydrodigoxin.

As noted before, although most antibodies are produced by methods analogous to that originally described by Smith et al. (5), the kits do differ in antibody titer, different methods of attaching the radio-label to either digoxin or a digoxin derivative and different methods of separating bound from free antigen. Any of these could cause the observed differences in specificity, as all of these factors depend on the strength of the equilibria between the antibody, the unlabeled antigen, and the labeled antigens, and the effect of the separation method on these interactions.

We thank Dr. G. A. Bruno (Squibb Institute for Medical Research), Mr. D. Holback (Corning Glass Works), and Dr. L. Lyle (Mallinckrodt Nuclear) for providing digoxin radioimmunoassay kits produced by their respective companies.

References

4. Clark, D. R., and Kalman, S. M., Dihydrodigoxin: a common me-
Lipoprotein Cholesterol in the Serum of Children, as Determined Independently by Two Different Methods

Sathanur R. Srinivasan, Ralph D. Ellefson, Carl F. Whitaker, and Gerald S. Berenson

We separately evaluated split specimens of serum for lipoprotein cholesterol in 38 five- to 14-year-old children, by the heparin/Ca\(^{2+}\) precipitation method and the ultracentrifugation/dextran sulfate precipitation method. Statistical analysis of the results indicated an excellent agreement (especially in \(\beta\) and \(\alpha\)-lipoprotein cholesterol values) between results by the two methods. The percentage of \(\alpha\)-lipoprotein cholesterol (mean ± SD: 38.6 ± 5.0) in children further confirmed the earlier observations that cholesterol derived from \(\alpha\)-lipoprotein in children constitutes a relatively greater part of the serum total cholesterol than is true of adults.

**Additional Keyphrases:** lipoproteins · polyanionic precipitation method · ultracentrifuge method · pediatric chemistry · screening

Because increased concentrations of certain serum lipoproteins relate to susceptibility to coronary artery disease, practical methods are greatly needed for quantitating serum lipoproteins in the general population, especially in children. Several such methods that have been proposed are based on complexing with polyanionic macromolecules (1–5) and are currently being used in clinical as well as population studies. Sulfated polysaccharides—i.e., heparin or dextran sulfate—are used because these materials react to form insoluble complexes with pre-\(\beta\)- and \(\beta\)-lipoproteins in the presence of certain divalent cations.

Recent studies indicate that the characteristics and concentrations of glycosaminoglycans, the lipoproteins in serum, and the divalent metal ions (e.g., Ca\(^{2+}\) or Mn\(^{2+}\)) are all critical for quantitative and selective precipitation of the lipoproteins (6, 7). Based on these observations, a simple and inexpensive procedure for quantitating the serum lipoproteins was developed (8, 9), the results of which correlated reasonably well with studies by analytical ultracentrifugation (10, 11). Although it gave similar values for adults (mostly men) when compared to results obtained with a heparin/Mn\(^{2+}\) precipitation method (12–14), the heparin/Ca\(^{2+}\) precipitation method consistently gave higher values for \(\alpha\)-lipoproteins in children (12, 15, 16). Therefore, we have attempted to compare values for serum lipoprotein cholesterol concentration obtained in children by our method (LSU method) with another method developed independently at the Mayo Clinic (Mayo method). The latter procedure, developed from the observations reported by Burstein et al. (17), is a combination of preparative ultracentrifugation and polyionic (dextran sulfate) precipitation that permits quantitation of cholesterol content of pre-\(\beta\)-, \(\beta\)-, and \(\alpha\)-lipoproteins by direct measurements.

**Materials and Methods**

**Serum**

Sera were collected in five different batches (March–July 1974) from 38 school children (18 boys and 20 girls), ages 5 to 14 years, who were among the 3524 children screened during the 1973–1974 school year as part of the Bogalusa Heart Study (Bogalusa, La.). Each child was asked to fast for 12–14 h and fasting compliance was determined by interview on the morning of the examination. Venous blood was collected and was allowed to clot. After centrifugation, sera were collected in tubes containing thimerosal, an antibacterial agent (Aldrich Chemical Co., Inc., Milwaukee, Wis. 53233), placed in a shipping box containing frozen packs, and sent by bus to New Orleans, where they were refrigerated at 4 °C overnight. The next day the samples were divided into two parts for analyses at LSU and at the Mayo Clinic. The samples were shipped airmail to the Mayo Clinic in containers packed with ice. Twelve of the 38 samples were randomly selected and the split sample included as blind duplicates.

**LSU Method**

**Serum cholesterol.** Serum cholesterol was determined with an AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, N. Y. 10591) according to the protocol developed by the Lipid Research Clinics in collaboration with the Center for Disease Control in Atlanta, Georgia. An isopropanol extract of the sample (0.2 ml of whole serum or \(\beta\) + pre-\(\beta\)-lipoprotein fraction) was used for the determination. We used a serum

---

1 Department of Medicine, Louisiana State University School of Medicine, 1542 Tulane Ave., New Orleans, La. 70112.
2 Department of Laboratory Medicine, Mayo Clinic, Rochester, Minn. 55901.
3 Direct correspondence to this author.

Received Sept. 26, 1977; accepted Oct. 28, 1977.