Digoxin Immunoassay with Cross-Reactivity of Digoxin Metabolites Proportional to Their Biological Activity

James J. Miller, Rudolph W. Straub, Jr., and Roland Valdes, Jr.

Our objective was to identify commercially available digoxin immunoassays whose cross-reactivity with digoxin metabolites paralleled the pharmacological activity of the metabolites. We measured the immunoreactivity of digoxigenin bis- and monodigitoxosides, digoxigenin, and dihydrodigoxin in four immunoassays and compared the immunoreactivities with pharmacological activities from studies involving whole-animal and receptor (Na,K-ATPase)-based assays. Correlation coefficients for comparisons of immunoassay reactivity and human heart receptor reactivities were: ACS, 0.96; TDx, 0.60; Stratus, 0.57; and Magic, 0.42. Comparison with other biological assays showed a similar trend. The major difference in metabolite cross-reactivities among the immunoassays was that of digoxigenin (ACS, 0.7%; TDx, 103%; Stratus, 108%; Magic, 153%), which has ~10% bioactivity relative to digoxin. Measured recovery of mixtures of digoxin and metabolites confirmed these findings. We conclude that the monoclonal antibody in the ACS digoxin assay closely mimics Na,K-ATPase in detecting digoxin and its metabolites. This finding provides a basis for developing therapeutic drug monitoring immunoassays capable of approximating the true pharmacological activity of a mixture of drug metabolites.

Indexing Terms: variation, source of/intern method comparison/ouabain receptor

Many drugs are metabolized to one or more fully or partially active components, and the blood often contains a mixture of the parent drug and its metabolites (1). The proportions of the parent drug and metabolites may vary throughout dosing and with changes in hepatic or renal function. Several drugs routinely assayed for therapeutic drug monitoring (TDM) purposes fall into this category, including carbamazepine, lidocaine, procainamide, quinidine, theophylline, tricyclic antidepressants, cyclosporine, and digoxin (1-4). Biological assays for TDM (5-7), which measure therapeutic or toxic effects on whole animals in vivo, physiological effects on target issues, or biochemical effects on the drug receptor, respond to a mixture of the drug and its metabolites in proportion to the sum of the pharmacological activity of each component present. Serum drug concentrations are typically measured by immunoassays. Immunoassays are capable of great analytical specificity; however, the relative responses of drug metabolites (cross-reactivities) are often greater than (or less than) the pharmacological activity of the metabolites. An immunochemical approach to solving this problem is to develop immunoassays for which the response would closely mimic a true measure of bioactivity. This concept requires developing antibodies with binding properties (e.g., affinities, kinetics) towards bioactive ligands (parent compound and active metabolites) proportional to the relative response of those ligands to their natural receptors. In selecting such antibodies, one must demonstrate equivalence of response in the immunoassay and biological assays to the individual metabolic components of the drug.

Digoxin can serve as a model to demonstrate these concepts. The pharmacological action of digoxin (and other digitals-related cardiac glycosides) is mediated by interaction with its receptor, the sodium pump (Na,K-ATPase; EC 3.6.1.37). Investigators have used receptor-binding assays (8, 9) and other receptor-based functional assays (10-12) to measure pharmacologically active digoxin and other cardiac glycosides in serum. Digoxin undergoes metabolism to various deglycated, reduced, and polar metabolites (13-17), the extent and magnitude of which varies greatly between individuals taking the drug (13, 14, 16). The pharmacological activities of some of these metabolites have been measured by using both animal models (18-21) and receptor-binding techniques (9, 22).

It has been suggested that if an endogenous ligand and a drug act by binding to the same cellular receptor, then antibodies against the drug may also bind the endogenous ligand (23). This phenomenon would be most likely to occur with antibodies that have binding sites very similar to the receptor ligand-binding site. This concept can be extended to active metabolites of drugs: If the receptor and antibody-binding sites were identical, then the binding affinities of a drug and its metabolites should be proportionately the same for the receptor and the antibody. An immunoassay for a drug involving such an antibody should give results similar to a receptor-based assay, even when active drug metabolites are present.

Our objective was to determine whether commercially available digoxin immunoassays have specificities similar to metabolite bioactivities and thus could reflect a truer measure of the pharmacological activity of digoxin and its active metabolites in serum. We compared reports of in vivo, receptor, and enzyme activity-based assays with the performance of four commercially available and widely used digoxin immunoassays for their
ability to measure digoxin and its metabolites in a manner reflective of the relative bioactivities of these analytes.

Materials and Methods

Materials. Digoxin (Dig) and digoxigenin (DG) were purchased from Sigma Chemical Co., St. Louis, MO. Digoxigenin bisdigitoxoside (DB), digoxigenin monodigitoxoside (DM), and dihydridigoxin (DHD) were generously provided by Burroughs Wellcome, Research Triangle Park, NC. Lyphochek Immunoassay Control Serum (three concentrations) was from Bio-Rad ECS Div., Anaheim, CA. ACS Digoxin (ACS) and Magic Digoxin RIA kits were provided by Ciba Corning Diagnostics, Medfield, MA. TDx Digoxin II kits were purchased from Abbott Labs., Abbott Park, IL. Stratus Digoxin kits were purchased from Baxter Diagnostics, Dearfield, IL. Primary rabbit anti-digoxin antiserum and 125I-labeled digoxin for the digoxin RIA were purchased from NEN Research Products, Boston, MA. Goat anti-rabbit antiserum for the digoxin RIA was from Sigma.

Assays. The ACS, TDx, Stratus, and Magic assays were performed according to the manufacturers' instructions. A previously described digoxin RIA (24) was also compared. The ACS assay involves a monoclonal antibody; the other assays have polyclonal antiserum.

Cross-reactivity studies. We prepared solutions of DB, DM, DG, and DHD at 0.025–0.05 mmol/L in methanol. For DB, DM, and DG, the exact concentrations were determined by their ultraviolet absorbance at 220 nm. We determined the molar absorptivity of Dig and DG in methanol at that wavelength to be $1.34 \times 10^4$ and $1.46 \times 10^4$ L·mol$^{-1}$·cm$^{-1}$, respectively. To measure DB and DM, we used the mean of these molar absorptivities, i.e., $1.40 \times 10^4$ L·mol$^{-1}$·cm$^{-1}$. For DHD, which does not absorb at 220 nm, the gravimetrically prepared concentration was assumed to be correct. We added these solutions to digoxin-free, digoxin-like immunoreactive factor-free plasma (no detectable digoxin immunoreactivity by any of the assays) and serially diluted solutions of individual metabolites as well as various mixtures of Dig and metabolites with the plasma. To construct response curves, we plotted the assay response at each concentration expressed as a percentage of the assay response for a sample with a concentration of zero analyte. The kinds of assay responses are as follows: ACS, relative light units; TDx, net polarization; Stratus, rate; Magic and RIA, 125I counts/min in the bound fraction. Cross-reactivity is expressed as the apparent digoxin concentration divided by the actual concentration of the cross-reactant times 100% at 50% assay response.

Results

Cross-reactivity of individual digoxin metabolites in immunoassays. Immunoassay response (displacement) curves for digoxin metabolites are shown in Fig. 1. The ACS assay shows a decrease in relative cross-reactivity, indicated by the shift toward higher concentrations on the displacement curves, as the terminal digitoxose sugars are sequentially removed from digoxin. Removal of digitoxose units had little effect on cross-reactivities in the TDx, Stratus, and Magic assays and on the digoxin-RIA (data not shown). The percent cross-reactivity at 50% assay response for these curves is summarized in Table 1.

Activity of digoxin metabolites in biological assays. We reviewed the literature for reports documenting the measurement of biological activity of digoxin metabolites; the results are summarized in Table 2. Note that several kinds of assays have been used, including: whole-animal studies monitoring some aspect of heart

![Fig. 1. Digoxin immunoassay response curves of the individual digoxin metabolites for various commercially available assays: O, Dig; •, DB; Δ, DM; A, DG; □, DHD.](Image)
or muscle function, inhibition of Na,K-ATPase, and competitive displacements of ouabain in a radioreceptor assay. We calculated the correlation coefficients of metabolite cross-reactivities in each assay (Table 1) with their reported bioactivity (Table 2) for each of the four biological assays that included data for DB and DM (9, 18, 20, 21). In the study by Lage and Spratt (18), which did not include DHD, a molar potency of 1% was assumed. Correlation coefficients for the ACS assay were all >0.94. Correlation coefficients for the other immunoassays were all <0.66. We chose to use the [3H]ouabain displacement from human heart ouabain receptors as the model for TDM of digoxin in humans. Fig. 2 shows the relation between metabolite immunoreactivities in the four immunoassays (from Table 1) and the metabolite molar potencies for displacement of ouabain from human heart receptor (Table 2, last row). The correlation coefficients are: ACS, 0.96; TDx, 0.60; Stratus, 0.57; Magic, 0.42.

Immunoreactivity of combined mixtures of digoxin metabolites. Because patients' sera contain a mixture of digoxin with its metabolites, we prepared a series of mixtures of Dig, DB, DM, and DG. DHD was not included because it reportedly has insignificant bioactivity and because it had insignificant cross-reactivity in all immunoassays we tested. These mixtures were assayed in the ACS, TDx, Stratus, and Magic digoxin assays. These results are listed in Table 3 and graphed in Fig. 3.

**Table 1. Cross-reactivity of digoxin metabolites in digoxin immunoassays.**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>ACS</th>
<th>TDx</th>
<th>Stratus</th>
<th>Magic</th>
<th>RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dig</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>DB</td>
<td>75</td>
<td>95</td>
<td>96</td>
<td>107</td>
<td>126</td>
</tr>
<tr>
<td>DM</td>
<td>47</td>
<td>83</td>
<td>83</td>
<td>108</td>
<td>123</td>
</tr>
<tr>
<td>DG</td>
<td>0.7</td>
<td>103</td>
<td>108</td>
<td>153</td>
<td>143</td>
</tr>
<tr>
<td>DHD</td>
<td>0.05</td>
<td>1.2</td>
<td>0.01</td>
<td>3.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* (Apparent digoxin in nmol/L divided by the metabolite concentration in nmol/L) × 100%.

**Table 2. Relative activity of digoxin metabolites in biological assays.**

<table>
<thead>
<tr>
<th>Effect</th>
<th>Measure</th>
<th>Animal</th>
<th>Tissue</th>
<th>DB</th>
<th>DM</th>
<th>DG</th>
<th>DHD</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxicity</td>
<td>LD₅₀</td>
<td>Mouse</td>
<td>Whole</td>
<td>75</td>
<td>68</td>
<td>21</td>
<td>—</td>
<td>18</td>
</tr>
<tr>
<td>Toxicity</td>
<td>Heart stop</td>
<td>Cat</td>
<td>Whole</td>
<td>—</td>
<td>—</td>
<td>53</td>
<td>&lt;4.2</td>
<td>19</td>
</tr>
<tr>
<td>Inotrop. +</td>
<td>Tension</td>
<td>Cat</td>
<td>Pap. mus.</td>
<td>—</td>
<td>—</td>
<td>67</td>
<td>—</td>
<td>19</td>
</tr>
<tr>
<td>Toxicity</td>
<td>Heart stop</td>
<td>Guinea pig</td>
<td>Whole</td>
<td>—</td>
<td>—</td>
<td>4.3</td>
<td>&lt;2.2</td>
<td>19</td>
</tr>
<tr>
<td>Inotrop. +</td>
<td>Amplitude</td>
<td>Guinea pig</td>
<td>Heart</td>
<td>—</td>
<td>—</td>
<td>10</td>
<td>—</td>
<td>19</td>
</tr>
<tr>
<td>Inotrop. +</td>
<td>Amplitude</td>
<td>Guinea pig</td>
<td>Heart</td>
<td>142</td>
<td>131</td>
<td>9.5</td>
<td>0.7</td>
<td>20</td>
</tr>
<tr>
<td>ATPase inhib.</td>
<td>P, formed</td>
<td>Guinea pig</td>
<td>Heart</td>
<td>85</td>
<td>80</td>
<td>8.4</td>
<td>2.2</td>
<td>21</td>
</tr>
<tr>
<td>Receptor</td>
<td>Displacement</td>
<td>Human</td>
<td>Heart</td>
<td>52</td>
<td>47</td>
<td>11</td>
<td>&lt;1</td>
<td>9</td>
</tr>
</tbody>
</table>

* Concentration causing 50% mortality.
  a Whole animal.
  c Not tested.
  d Positive inotrop effect.
  e Papillary muscle.
  f Of recorded heartbeat.
  g Ouabain-receptor binding measured by displacement of [3H]ouabain binding.

**Discussion**

In this study we used digoxin immunoassays as a paradigm to establish whether the antibodies currently available for measuring digoxin would measure digoxin metabolites in a manner proportional to their reported biological activity. We compared the immunoassay results with various bioassay responses to the major metabolites of digoxin.

We found the TDx, Stratus, Magic, and RIA assays to be relatively insensitive to the removal of terminal digitoxose residues from digoxin, with cross-reactivities near or >100%. The antibody used in the ACS assay, however, was sensitive to the presence and number of digitoxose sugars, with cross-reactivities decreasing as the sugars are sequentially removed. None of the assays had significant cross-reactivity with DHD. The greatest difference between the ACS assay and the other assays was in the significant cross-reactivity with digoxigenin (0.7% in the ACS assay and 103–153% in the other assays).

Metabolite cross-reactivities for the ACS (25, 26), TDx (26, 27), and Stratus (26) assays have been reported to be higher than our results (Table 1). The reason for this discrepancy is that each of these reports expressed the concentrations of metabolites and the apparent digoxin measured on a weight basis (μg/L). Cross-reactivity is most often expressed as the concentration of cross-reactant divided by the measured concentration of the analyte times 100%. For cross-reactivity values to truly represent the relative response in the assay, the concentration terms in this equation must be expressed on a molar basis. Expressing concentrations on a weight basis introduces significant error in the calculated cross-reactivity if the molecular masses of the cross-reactant and the analyte are significantly different, as is the case with digoxin and its deglycosylated metabolites. Cross-reactivity expressed on a weight basis can be converted to molar cross-reactivity by multiply-
Fig. 2. Comparison of relative immunoreactivity with relative radioreceptor activity (human heart) for digoxin and its metabolites: +, ACS; Δ, TDx; ○, Stratus; □, Magic.

ing by the ratio of molecular masses of the cross-reactant and the analyte. The molecular masses of digoxin and its metabolites are: Dig, 781; DB, 651; DM, 521; and DG, 391 Da. Therefore, the conversion factors for DB, DM, and DG to Dig are 0.834, 0.667, and 0.500, respectively. Our cross-reactivity data are thus comparable with published cross-reactivity data (25–27) when the latter are expressed on a molar basis.

Our search for the types of assays used to measure digoxin and metabolite bioactivity revealed several (9, 18–21). The results, summarized in Table 2, show several important trends: (a) bioactivity decreases as sugars are removed from digoxin; (b) the bioactivity of the deglycosylated metabolites of digoxin are most often reported to be less than the activity of digoxin (except for the report by Dzimir and Fricke (20)); (c) the bioactivity of DG is usually reported to be near 10% relative to Dig (except for the studies in cats reported by Brown et al. (19)); and (d) the bioactivity of DHD is insignificant, at least relative to heart-related effects. Although the positive inotropic effect and (or) the toxic effect of the metabolites in humans in vivo would be the best measure of their bioactivity, these data are not at present available. The closest model in humans seems to be the human heart ouabain-receptor work by Bednarczyk et al. (9). Consequently, we compared the immunoassay cross-reactivity results with the radioreceptor studies of the human heart tissue system. We tested digoxin metabolites individually and also in proportional mixtures (as would be seen in human serum in treated patients (13–17)) because the net antibody reactivity of a group of simultaneously reacting ligands is not always additive (28, 29).

In experiments with individual digoxin metabolites added to serum, our data (Fig. 2) demonstrate a good correlation between the response of the antibody used in

<table>
<thead>
<tr>
<th>Table 3. Apparent digoxin in mixtures of metabolites.</th>
</tr>
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<tbody>
<tr>
<td><strong>Conc In mixture, nmol/L</strong></td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td><strong>Sample</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
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<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total concentration of Dig, DB, DM, and DG.

<sup>b</sup> Calculated radioreceptor assay activity (human heart) from the molar potencies (see last row of Table 2): RRA = [Dig] × 1.00 + [DB] × 0.52 + [DM] × 0.47 + [DG] × 0.11.
the ACS assay and the response of the digoxin metabolites in the human heart ouabain-receptor displacement assay (9). In the ACS assay DB is slightly above and DG is slightly below the line of identity (Fig. 2) relating immunoactivity with ouabain receptor activity. For the other assays the immunoactivities of DB, DM, and DG are much greater than the receptor activity of these metabolites.

In other experiments the proportions of digoxin metabolites were varied to simulate proportions that might be present in a patient; these results also indicate that the ACS immunoassay responds like the receptor-based assay (Fig. 3; slope, 1.03; y-intercept, 0.03; r, 0.862). The other assays showed significantly higher measured apparent digoxin and poorer correlations with the human heart tissue radioreceptor assay (Fig. 3). In spite of the positive bias in the TDx, Stratus, and Magic assays, these assays gave similar results, as predicted because of their similar high cross-reactivities with digoxin metabolites.

Traditionally, the antigenic conjugates of digoxin with protein have been prepared by linkage at the terminal sugar (30). Antibodies generated against these conjugates, such as those used in the TDx, Stratus, and Magic assays, are usually insensitive to structural changes around the sugars. Several investigators have reported the production of antibodies with increased specificity for digoxin (31–35). However, many of these antibodies that show great specificity for digoxin have little cross-reactivity with DB, DM, and DG, so the cross-reactivities are not in proportion to the bioactivity of these metabolites. Wahyono et al. (34, 35) describe one monoclonal antibody (4B10) that has a pattern of cross-reactivity very similar to that of the antibody used in the ACS assay, and another monoclonal antibody (6C9) that has metabolite cross-reactivities even closer to those in the human heart radioreceptor assay (9): DB, 66%; DM, 42%; and DG, 24%. To the best of our knowledge, neither of these antibodies (31–35) is currently used in commercially available digoxin assays.

Other metabolites of digoxin have been identified (15, 16, 36). Quantitatively, the most abundant of these are the polar metabolites, predominantly glucuronide and sulfate conjugates of 3α-(epi)-digoxigenin (36). Because of the limited availability of these compounds, little is known about their bioactivity or cross-reactivity. After administering [3H]Dig to volunteer patients, Gault et al. (16) estimated that the cross-reactivity of polar metabolites in patients’ sera varied between 25% and 85% (mean 33%). Given this degree of cross-reactivity and the probability that these metabolites are relatively inactive, polar metabolites may contribute substantially to discrepancies between the immunoactivity and pharmacological activity in serum samples.

In conclusion, we have found that one digoxin immunoassay (ACS) closely approximates the response of a human heart receptor-based assay when DB, DM, and DG are present, whereas other immunoassays (TDx, Stratus, and Magic) do not. This approach provides a "bioactive average" response that may set the standard for future digoxin immunoassays. This latter premise needs to be studied in patients undergoing digoxin therapy under a variety of controlled conditions. Our findings with the digoxin model are important because they demonstrate that the concept of developing antibodies that mimic the ligand response to natural receptors is apparently achievable. Extension of this principle to other drugs with active metabolites may represent a marked improvement in TDM over current immunoassays for measuring drugs in serum.

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References
18. Lage GL, Spratt JL. Structure–activity correlation of the