Measurement of Digoxin by Radioimmunoassay

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ABSTRACT

A sensitive, specific and precise procedure for the measurement of digoxin by radioimmunoassay is presented. The method is rapid, convenient and highly reliable for this very important measurement. Studies designed to evaluate the validity and reproducibility of the assay are presented and discussed.

Introduction

The radioimmunoassay (RIA) of digoxin in serum or plasma, although a relatively recent technique, has received much attention in the literature since the original report. RIA is presently the most convenient method available to the clinical laboratory for the accurate measurement of digoxin levels in biological fluids, because of its sensitivity, rapidity and simplicity.

In the past, lack of convenient methodology for the detection and management of toxic dose levels in individual patients complicated administration of therapeutically effective quantities of digitalis glycosides. A narrow range exists between beneficial and toxic levels of the digitalis glycosides. Digitalis is excreted slowly, leading to cumulative effects and the danger of poisoning from repeated excess doses. In addition, clinical prediction of total body digoxin stores is complicated by variation in absorption of oral doses and variation in non-renal excretion.

The clinical value of the measurement of serum digoxin levels by RIA is greatest when carefully interpreted in conjunction with the clinical status of the patient and electrocardiographic findings. Serum electrolytes, renal function, age and the nature and severity of the underlying heart disease are variables which must be considered when assessing digoxin toxicity.

The routine availability of accurate serum digoxin levels has become a valuable adjunct to patient care for the following reasons: (1) the margin of safety between therapeutic and toxic doses of the drug is narrow; (2) many of the signs and symptoms of digitalis excess may be
mimicked by underlying cardiac disease; and (3) standard doses of this drug may result in toxicity when renal function is impaired or may be ineffective if malabsorption is present. Digoxin levels are helpful when an adequate history of digitalis therapy cannot be obtained. In addition, since the relationship between the steady-state plasma level and the digoxin dose is proportional, digoxin levels can be used to guide dose adjustment.

This report details studies designed to evaluate the performance characteristics of commercially available reagents for the measurement of serum digoxin employing a solid phase RIA technique.

Principle

The Immo-Phase™* digoxin radioimmunoassay is a solid-phase radioimmunoassay procedure. The goat antiserum to digoxin as provided in the kit is chemically immobilized to porous glass particles (approximately 1 micron in size) with relatively high density. A sample of patient serum or digoxin standard and a known amount of 125I-labeled digoxin derivative are placed in a test tube containing a known quantity of glass-bound antiserum suspended in a buffer solution. During the specified incubation period, the 125I-labeled digoxin derivative and the unlabeled digoxin present in the patient serum or standards compete for the available antibody binding sites on the glass-bound antiserum. After incubation, the 125I-labeled digoxin derivative and unlabeled digoxin not reacting with digoxin antibody are separated from the antibody-bound digoxin by centrifugation. As the concentration of unlabeled digoxin in standards or patient specimens increases, the amount of labeled digoxin bound to the glass-bound antiserum decreases, and vice versa. If the concentrations of labeled digoxin and antibody are constant, by using several known concentrations of unlabeled digoxin one can generate a standard curve which can be employed for quantitating unknown specimens.

Materials and Methods

The Corning kit contains the digoxin standards, 125I-labeled digoxin, and glass-bound antiserum to digoxin ready to use as described.

Digoxin Standards

Standard digoxin solutions of concentrations of 0.5, 1.0, 2.5 and 5.0 ng per ml prepared employing digoxin-free normal human serum as a protein source were used for standardizing the assay.

Digitoxin Standards

Standard solutions of digitoxin for the evaluation of cross-reactivity of digitoxin in the digoxin assay were prepared from a preparation of crystalline digitoxin. A stock solution (100 µg per ml) was prepared by adding 10 mg of digitoxin to a 100 ml volumetric flask and diluting to volume with reagent grade methanol. A further dilution of the digitoxin was prepared (1 µg per ml) by diluting 1.0 ml of the original preparation to 100 ml with methanol. Working concentrations of digitoxin were prepared by pipeting 5.0 ml (5 µg) of the second dilution (1 µg per ml) into a 15 x 100 glass test tube and evaporating the methanol to dryness with a gentle stream of nitrogen.

Following removal of the methanol, 5.0 ml of digitoxin-free normal human serum were added to the dried digitoxin preparation and allowed to stand for 30 minutes at room temperature to allow for complete solution (1000 ng per ml). This preparation was then further diluted tenfold with digitoxin-free normal serum to provide a digitoxin concentration of 100 ng per ml.

* Coming Biological Products, Medford, MA.
† Calbiochem, San Diego, CA.
ng per ml. Digitoxin concentrations of 50, 25, 12.5 and 6.25 ng per ml were then prepared by serial dilution of the 100 ng per ml stock with digitoxin-free normal human serum.

ANTI-DIGOXIN SERUM

The goat anti-digoxin serum employed for the procedure was supplied covalently linked to porous glass particles in phosphate buffered saline containing bovine serum albumin in premeasured amounts in 12 × 75 mm polystyrene tubes.

RADIOIODINATED DIGOXIN

The ¹²⁵I-tyrosine methyl ester of digoxin in phosphate buffered saline containing bovine serum albumin was ready for use as outlined in the next section.

RADIOIMMUNOASSAY PROCEDURE

The 12 × 75 mm polystyrene tubes containing the glass-bound antiserum were centrifuged at 1400 to 1600 × g for 10 minutes at room temperature to concentrate the glass-bound antiserum in the bottom of the tubes. Tubes were marked for duplicate zero (digoxin-free normal human serum), 0.5, 1.0, 2.5 and 5.0 ng per ml standards. Additional duplicate tubes were marked for each control and patient specimen to be analyzed. Aliquots (200 µl) of the individual standards, patient specimens and control sera were added to the appropriately marked tubes followed by the addition of 50 µl of the ¹²⁵I-digoxin to each tube. Duplicate total count tubes were prepared by adding 200 µl of digoxin-free normal human serum and 50 µl of ¹²⁵I-digoxin to empty 12 × 75 mm tubes containing a volume of phosphate buffered saline plus bovine serum albumin equal to that in the tubes containing glass-bound antiserum.

All of the tubes were then vortexed gently for three to five seconds and incubated at room temperature for 30 minutes. Following the incubation period, all of the tubes (with the exception of the total count tubes) were centrifuged at 1400 to 1600 × g for 10 minutes at room temperature, removed from the centrifuge, and decanted to separate the radioactivity in the supernatant from that bound to the glass-bound antiserum particles which were in the bottoms of the tubes. All of the tubes were blotted on absorbent paper to remove residual radioactivity from the lips of the tubes and counted in an automatic gamma scintillation spectrometer† for 0.5 minutes each.

CALCULATIONS

The duplicate counts for the total counts, standards and patient specimens were averaged. The standards, patient specimens and controls were calculated as a percentage of the zero standard in order to determine the percent binding to the antiserum for each of the other standards, patient specimens and controls. The calculations were performed as follows:

\[
% B_0 = \frac{\text{Av. counts of std. or spec.}}{\text{Av. counts of zero std.}} \times 100
\]

The standards were subjected to linear regression analysis of the logit transformation of the percent bound versus the log of the digoxin concentration. From this analysis, the slope and the y-intercept of the standard line were obtained. Using the formula for a straight line (where \(y = \text{logit of the percent bound and } x = \text{log of the digoxin concentration}\), the concentration of digoxin in each patient specimen and control was computed.

† Model 1285, Searle Analytic, Inc.
DIGOXIN, ng/ml

Figure 1. Standard curve for the digoxin assay. The percent antibody-bound 125I-digoxin is plotted as a function of the digoxin concentration (ng per ml). The plot was prepared using the logit transform method (see text) and special graph paper (Codex Book Co., Norwood, MA) which plots the logit of the percent bound as a function of the log of the digoxin concentration.

Results

Sensitivity and Specificity of the Anti-Digoxin Serum

In figure 1 is shown a typical standard line obtained with crystalline digoxin when plotted by the logit method. When performed employing a computer using linear regression analysis and statistical evaluation of significance, the logit transformation is capable of making a confident, reliable estimate of assay sensitivity which is generally at a lower concentration of analyte than might be estimated by visual examination of the plotted data. With this digoxin assay, the lower limit of detection has generally been 0.1 to 0.3 ng per ml.

Evaluation of the cross-reactivity of digitoxin in the digoxin assay is illustrated in figure 2. Approximately 40 times as much unlabeled digitoxin (100 ng per ml) as unlabeled digoxin (2.5 ng per ml) was required to give 50 percent displacement of binding of 125I-digoxin to the glass-bound antiserum. The cross-reactivity of the digoxin antiserum for digitoxin is thus only 2.5 percent. Although this value is low, normal therapeutic levels of digitoxin are much higher than for digoxin. Because it is possible that a toxic level of digitoxin would appear to be a normal therapeutic level of digoxin, it is necessary either to know which drug is being used or to run separate tests for digoxin and digitoxin on each specimen.

Several reports have appeared in the literature concerning the interference by spironolactone* in the assays for digoxin. An attempt has been made to determine whether or not this drug might interfere with the digoxin assay described by determining digoxin levels in a group of five volunteers who received 25 mg of spironolactone three times per day for two days as compared to a control group of five individuals who received no medication. All subjects who received spironolactone had “digoxin” levels of 0.2 to 0.3 ng per ml, whereas those in the control group had non-detectable digoxin results. Similar observations were made by Huffman and by Ravel employing different antisera for the measurement of digoxin. Huffman concluded that canrenone, a metabolite of spironolactone, and not the parent compound, was apparently the compound responsible for the interference noted in his studies on patients receiving spironolactone alone or together with standard doses of digoxin.

The specificity of the digoxin antiserum was further evaluated by making serial dilutions of an elevated patient specimen with a digoxin-free normal human serum. The results of this study, shown in figure 3, indicate that the digoxin in the patient specimen reacted

* Aldactone® Searle.
similarly in the assay to the crystalline digoxin used as the standard in the assay. This type of parallelism between the amount measured and the quantity expected is an accepted procedure for validation of an RIA.

**PRECISION**

Data documenting the consistency of the digoxin standard curve over a period of 11 working days are presented in table I. These data represent a summary of the $^{125}$I-digoxin bound to the antibody at various concentrations of digoxin. The percent of the total counts bound ($B/T \times 100$) and the percent of the counts bound relative to the zero standard ($B/B_0 \times 100$) both demonstrated acceptable precision as indicated by the statistical data summarized in table I. In these calculations, $B$ is the counts of $^{125}$I-digoxin bound to antiserum in each tube, $T$ is the total counts used and $B_0$ is the counts bound in the zero standard.

Inter-assay variation of the assays was routinely monitored through use of two different serum pools in each assay. These pools were established at levels which approximated an upper therapeutic level and a toxic level for digoxin. In table II is represented a summary of the data collected over a one-month period of time during which the therapeutic (normal) and toxic (elevated) pools were analyzed at the beginning of each assay and following every tenth patient specimen. The numbered positions indicated in table II refer to the sequence of the controls within the individual assays. The estimates of precision for both pools demonstrated excellent overall control for the digoxin assay. In addition, no significant differences were noted for either pool relative to the various positions within the individual assays.

Lack of consistency between specimens assayed at the beginning and end of an assay containing a large number of specimens is a problem frequently encountered with the RIA technique. This problem is easily evaluated on a routine basis by positioning standards and control samples at the beginning, middle and end of large assays. If the assay demonstrates consistent standard and control values, irrespective of position within the assay, the assay can be considered valid for unknown specimens, irrespective of
position. The data in Table II indicate that the two quality control pools were not affected quantitatively relative to position within the assay. Further documentation of the consistency between the beginning and the end of the assay is presented in Table III. These data illustrate the effect of positioning (beginning versus end) of the standard curves on the counting rates of the individual standards. They show that the standard curves evaluated on five consecutive days demonstrated acceptable agreements with the general characteristics of the curves at the beginning of the individual assays as compared to the curves at the ends of the assays. This is an important performance parameter that should be evaluated for any RIA, regardless of the substance being measured.

In Table IV is contained a summary of a statistical evaluation performed employing the standard Student's t-test on the data accumulated on replicates of 100 patient specimens to demonstrate the reproducibility of the assay with actual specimens from the clinical service. These data clearly document that the assay is capable of providing very consistent measurements over the range of digoxin levels required for evaluating sub-therapeutic, therapeutic and toxic levels.

**DISTRIBUTION OF PATIENT VALUES**

The routine availability of timely, accurate, precise digoxin levels has enabled the clinician to control more accurately the administration of digoxin for proper control of digoxin therapy. In Figure 4 is shown the distribution of digoxin levels measured on 1290 specimens submitted to the laboratory for the quantitation of digoxin over a one-month period. Of the 1290 specimens assayed, 69.5 percent (896) were assayed at levels considered to be within the usual therapeutic range (0.5 to 2.0 ng per ml), 10.4 percent (134) were assayed at levels less than 0.5 ng per ml, 14.1 percent demonstrated levels between 2.0 and 3.0 ng per ml, and 6 percent (78) were assayed at

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**TABLE I**

Reproducibility of the Standard Curve for the Digoxin Assay

| Digoxin Standard (ng per ml) | Percent of Total Counts Bound (B/T x 100) Mean ± S.D. | Percent of Counts Bound Relative to Zero Standard (B/B₀ x 100) Mean ± S.D. | CV %
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>71.5 ± 2.05</td>
<td>2.9</td>
</tr>
<tr>
<td>0.5</td>
<td>85.9 ± 1.89</td>
<td>2.2</td>
</tr>
<tr>
<td>1.0</td>
<td>73.1 ± 2.19</td>
<td>3.0</td>
</tr>
<tr>
<td>2.5</td>
<td>44.8 ± 1.36</td>
<td>3.0</td>
</tr>
<tr>
<td>5.0</td>
<td>30.1 ± 1.68</td>
<td>5.6</td>
</tr>
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</table>

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**TABLE II**

Effect of Position of the Normal and Elevated Controls on the Precision of the Digoxin Measurement

<table>
<thead>
<tr>
<th>Position</th>
<th>Normal Control</th>
<th>Elevated Control</th>
</tr>
</thead>
</table>
|          | N   | Mean Digoxin (ng per ml) | S.D. | CV % | N   | Mean Digoxin (ng per ml) | S.D. | CV %
| # 1      | 30  | 1.54                     | 0.10 | 6.5  | 30  | 3.01                     | 0.16 | 5.3  |
| # 2      | 30  | 1.50                     | 0.11 | 7.3  | 30  | 2.94                     | 0.18 | 6.1  |
| # 3      | 30  | 1.52                     | 0.11 | 7.2  | 30  | 2.90                     | 0.16 | 5.5  |
| # 4      | 30  | 1.48                     | 0.11 | 7.4  | 30  | 2.88                     | 0.16 | 5.6  |
| # 5      | 30  | 1.48                     | 0.10 | 6.8  | 30  | 2.88                     | 0.18 | 6.3  |
TABLE III
Effect of Position (Beginning Vs. End) of the Standard Curve on the Standardization of the Digoxin Assay

<table>
<thead>
<tr>
<th>Time</th>
<th>Position</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
<th>2.5</th>
<th>5.0</th>
<th>Slope</th>
<th>Curve Characteristics</th>
<th>Correlation Coefficient (r)</th>
<th>Y-Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td><strong>Beginning</strong></td>
<td>91.22</td>
<td>80.13</td>
<td>68.20</td>
<td>41.27</td>
<td>28.31</td>
<td>-2.8352</td>
<td>0.9964</td>
<td>1.0826</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>End</strong></td>
<td>91.00</td>
<td>77.27</td>
<td>68.40</td>
<td>41.12</td>
<td>28.08</td>
<td>-2.6348</td>
<td>0.9954</td>
<td>0.9830</td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td><strong>Beginning</strong></td>
<td>92.00</td>
<td>79.47</td>
<td>68.08</td>
<td>42.27</td>
<td>28.28</td>
<td>-2.7111</td>
<td>0.9981</td>
<td>1.0189</td>
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<tr>
<td></td>
<td><strong>End</strong></td>
<td>92.08</td>
<td>79.99</td>
<td>65.66</td>
<td>41.89</td>
<td>28.89</td>
<td>-2.6817</td>
<td>0.9957</td>
<td>0.9926</td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td><strong>Beginning</strong></td>
<td>87.59</td>
<td>73.95</td>
<td>60.51</td>
<td>37.76</td>
<td>26.71</td>
<td>-2.5426</td>
<td>0.9957</td>
<td>0.8545</td>
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</tr>
<tr>
<td></td>
<td><strong>End</strong></td>
<td>87.25</td>
<td>71.55</td>
<td>61.75</td>
<td>39.12</td>
<td>26.17</td>
<td>-2.4160</td>
<td>0.9985</td>
<td>0.8172</td>
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</tr>
<tr>
<td>Day 4</td>
<td><strong>Beginning</strong></td>
<td>87.18</td>
<td>78.33</td>
<td>63.87</td>
<td>38.70</td>
<td>26.77</td>
<td>-3.0086</td>
<td>0.9918</td>
<td>1.1359</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>End</strong></td>
<td>87.56</td>
<td>77.49</td>
<td>63.94</td>
<td>42.06</td>
<td>27.44</td>
<td>-2.8078</td>
<td>0.9967</td>
<td>1.1020</td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td><strong>Beginning</strong></td>
<td>87.23</td>
<td>72.98</td>
<td>63.22</td>
<td>38.76</td>
<td>25.33</td>
<td>-2.5908</td>
<td>0.9980</td>
<td>0.8865</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>End</strong></td>
<td>86.98</td>
<td>74.33</td>
<td>63.63</td>
<td>38.12</td>
<td>25.20</td>
<td>-2.7325</td>
<td>0.9978</td>
<td>0.9503</td>
<td></td>
</tr>
</tbody>
</table>

levels greater than 3.0 ng per ml, a level at or above which signs and symptoms of toxicity are generally observed. These data tend to support the fact that the majority of the patients receiving digoxin are controlled fairly well within the therapeutic range and that the percentage of patients presenting with toxic levels of digoxin is relatively small. The number of patients with digoxin levels below the usual therapeutic range was surprisingly high. This may be in part due to an observation in elderly patients who frequently are less regular in maintaining a routine schedule for taking medication.

Discussion

The radioimmunoassay technique described is an excellent method for the determination of digoxin by the clinical chemistry laboratory. The rapidity, sensitivity and specificity characteristics of the method render it useful both as an aid to the clinician in arriving at accurate management decisions and as a tool for investigators studying the pharmacodynamics of digoxin.

Sources of Error

Serum or plasma collected by standard procedures may be used in this assay. If plasma samples are stored, they should be fresh, not previously frozen. Serum samples may be stored between 2° and 8° for assay within 48 hours or frozen for longer periods.
within-run precision of duplicate measurements of digoxin levels (ng/ml) in 100 serum specimens

<table>
<thead>
<tr>
<th>Summary of Statistical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of observations (n)</td>
</tr>
<tr>
<td>Sum of differences (Σd)</td>
</tr>
<tr>
<td>Mean difference (d)</td>
</tr>
<tr>
<td>Standard deviation of mean difference (s)</td>
</tr>
<tr>
<td>Student’s t-test (test of significance)</td>
</tr>
</tbody>
</table>

Test of Correlation
- Correlation coefficient (r) = 0.9911
- Slope of line = 0.9700
- Y-intercept = 0.0465
- Equation of regression line = y = 0.9700x + 0.0465

Periods of storage. Specimens must be free of particulate matter such as red cells, fibrin strands or insoluble proteins, because they may interfere with the assay. A blood sample should be drawn at least six hours after the most recent oral dose and two to three hours after the last intravenous dose in order to obtain an assay value that approximately reflects the steady-state physiological level and avoids the initial absorption maximum.11

Patients receiving digitoxin medication should not be assayed with the materials described for the digoxin procedure. To provide an accurate measurement or to identify the type of digitalis medication, separate, specific assays for digitoxin and digoxin should be employed. Clinicians should be advised that standard doses of spironolactone may elevate the level of digoxin measured by 0.2 to 0.3 ng per ml.

Résumé of Clinical Interpretations

Serum samples processed as described are generally considered toxic if digoxin levels are greater than 2.0 ng per ml. Therapeutic levels of serum digoxin have been reported as falling within a range of 0.7 to 2.3 ng per ml. Although some overlap occurs between therapeutic and toxic levels, toxicity is unlikely to occur below a concentration of 2.0 ng per ml.2 Studies of serum digoxin levels by several investigators are summarized in table V.

One of the most important single factors predisposing to digoxin toxicity has been impaired renal function. Mean blood urea nitrogen concentration and incidence of uremia are both significantly higher in toxic hospitalized patients than in nontoxic.9 Toxic patients tend to be older, a finding which may well exert its influence at least in part through diminished glomerular filtration rate and hence digoxin excretion.4 A high incidence of renal functional impairment was also evident in digoxin-toxic patients studied prospectively.1

Significantly higher values of mean serum digoxin levels have been found for neonates and infants,8 2.0 ± 0.9 (mean ± S.D.) ng per ml, compared to adults, 1.3 ± 0.4 (mean ± S.D.) ng per ml. The higher values in children are apparently related to the administered digitoxin dosages which were substantially larger than those used in adults on a basis of milligrams per kilogram of body weight. The higher values observed in the 17 children studied did not, however, produce cardiac rhythm disturbances suggesting digoxin intoxication. The same study found that fetal umbilical cord concentrations of digoxin were smaller than those in maternal venous blood in each of seven women receiving digitalis during pregnancy.
References


