Reduced Variation of Tracer Binding in Digoxin Radioimmunoassay by Use of \([^{125}\text{I}]\)-Labeled Tyrosine-Methyl-Ester Derivative: Relation of Thyroxine Concentration to Binding

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Between-sample variation in tracer binding in the \([^{125}\text{I}]\)-labeled digoxin radioimmunoassay was investigated with two tracers, 3-O-succinyl-digoxigenin--\([^{125}\text{I}]\)-labeled tyrosine and \([^{125}\text{I}]\)-labeled tyrosine-methyl-ester–digoxin. Digoxin-free serum samples having various concentrations of thyroxine were assayed with both tracers. The percentage of tracer bound when the samples were assayed with the first-mentioned tracer was increased significantly for the low thyroxine groups when compared to the normal \((P < 0.001)\) or the high thyroxine groups \((P < 0.05)\). Little difference existed when the latter tracer was used. There was variation in tracer binding when serum from dogs dosed with thyroid tropin was assayed with the first tracer, but there was little variation with the second. Tracer binding may be influenced by thyroxine-binding proteins. Variation in tracer binding appears to be reduced when \([^{125}\text{I}]\)-labeled tyrosine-methyl-ester–digoxin is used.

Additional Keywords: 3-O-succinyl-digoxigenin--\([^{125}\text{I}]\)-labeled tyrosine • variation, source of

The radioimmunoassay for digoxin has become widely used as an aid in the care of patients receiving digoxin therapy. There has, however, been a considerable amount of controversy regarding the value of information on serum digoxin concentrations in distinguishing toxic from nontoxic patients, as well as in determining a patient’s therapeutic status (1–3). Problems with assay methodology may contribute to differences regarding the significance of serum concentrations.

The digoxin radioimmunoassay can be accomplished with use of either \(^3\text{H}\) or the \([^{125}\text{I}]\). The \([^{125}\text{I}]\) method is often used in clinical and research settings, primarily because there is no interference by quenching. As a result, much investigation has centered on the \([^{125}\text{I}]\) assay. The relationship of albumin concentration to the binding of the \([^{125}\text{I}]\) tracer has been studied (2, 4). The variability of results obtained for different serum samples containing the same concentrations of digoxin has been shown (5). One group of investigators has shown differences between standard curves prepared with use of different normal sera (6).

Probably the most perplexing problem involved with the \([^{125}\text{I}]\) method is the variation in the binding of the tracer and thus in the height and slope of the standard curves that are prepared in various normal sera. These “unknown plasma factors” (6) can result in errors when the serum used for the standard curve differs in nonspecific tracer binding effects from the samples.

Two structurally different \([^{125}\text{I}]\)-labeled digoxin derivatives are in use in the digoxin radioimmunoassay. The one most frequently present in commercial digoxin kits is 3-O-succinyl-digoxigenin–\([^{125}\text{I}]\) tyrosine (SDT), in which iodinated succinyl-tyrosine is substituted for the three digitoxose sugars in the digoxin molecule. Recently, \([^{125}\text{I}]\)-labeled tyrosine-methyl-ester–digoxin (TME) has been used. Although the exact structure is in doubt, the \([^{125}\text{I}]\)-labeled tyrosine-methyl-ester is attached to the terminal digitoxose residue. Thus, all sugars remain in place.

In this study, the variation in binding of the two digoxin derivatives with different normal sera was investigated as well as serum factors that could possibly produce errors in the results obtained with the \([^{125}\text{I}]\) method.

Materials and Methods

Reagents. 3-O-Succinyl-digoxigenin–\([^{125}\text{I}]\)-labeled tyrosine and \([^{125}\text{I}]\)-labeled tyrosine-methyl-ester–digoxin were obtained from commercial suppliers. Albumin-containing buffer was prepared by adding 12.1 g of tris(hydroxymethyl)aminomethane and 2.5 g of bovine serum albumin (Fraction V) to 1 liter of distilled water and adjusting the pH to 7.4 with glacial acetic acid. Barbital buffer was prepared by adding 1.57 g of sodium barbital, 7.65 g of NaCl, and 0.97 g of sodium acetate to 1 liter of water and adjusting the pH to 7.4 with 1 mol/liter HCl.

A charcoal suspension was prepared by mixing 750 mg of charcoal (Carbon Decolorizing Alkaline–Norit A) with 120 ml of barbital buffer.

Preparation of antiserum. Antiserum to digoxin was obtained by immunizing a rabbit with a digoxin conjugate to human serum albumin (produced by the periodate oxidation method; five molecules of digoxin per molecule of human serum albumin) mixed in acrylamide gel and homogenized with a small amount of phosphate buffer, pH 7.4 (7). About 100 μg of the conjugate was injected intramuscularly twice a week for two weeks and once a week for three weeks. Subsequently, injections were given about every two weeks for a
month and serum was then collected from the rabbit. Cross reactivity of the antiseraum with 1000 µg/liter of various substances was as follows: progesterone (14%), 17α-estradiol (5%), 17β-estradiol (3%), testosterone (<1%), cortisol (3%), and spironolactone (5%).

Procedures. A 100-µl sample of serum was pipetted into a polystyrene tube, and 1 ml of the albumin-containing buffer and 50 µl of the radioactive digoxin derivative were added to the tube. Appropriate amounts of a solution of digoxin in ethanol/water (30/70 by vol) were used in the preparation of standards. The antiseraum was diluted 110-fold with the albumin-containing buffer, 50 µl of the dilution was added to each tube, and the contents of the tubes were mixed on a vortex-type mixer and incubated at room temperature for 30 min. Then 1 ml of the charcoal suspension was added, to separate bound from free molecules. (Charcoal adsorbed over 95% of the tracer when no antiseraum was present.) After a 3-min incubation the tubes were centrifuged at 3000 × g for 2 min at 4°C. The supernates were decanted into clean plastic tubes, and both they and the charcoal were counted to allow estimation of total counts per tube. Counting was done in a Packard Auto-Gamma Scintillation Spectrometer, Model 5986 with multi-channel analyzer (Packard Instrument Co., Downers Grove, Ill. 60515) that enables counting from 0.020 to 0.042 mEV.

For each sample, the percentage of the tracer bound by the antibody was calculated by dividing the counts in the supernatant fluid by the total counts in the original tube (counts in the supernate plus counts in the charcoal). Each sample was assayed in duplicate and means were calculated. Only those duplicates that differed by ≤±3% from each other were acceptable.

Serum samples having a wide range (<10 to >200 µg/liter of thyroxine concentrations were studied. These samples were assayed according to the procedure described above and the percentage bound was determined for each. Patients' charts were examined, to avoid using samples that contained digoxin, digitoxin, steroid drugs, or other drugs that were likely to cross-react with the antiseraum. Serum samples were assayed by use of both the SDT and the TME derivatives.

To study the effects of changing thyroxine status while other factors were controlled, we injected beagle dogs intramuscularly on three consecutive days with 10 int. units of thyrotropin. Blood samples were drawn two days before, immediately before, and 6 h after each injection, as well as on several days after the injections. The sera from these samples were then assayed to determine the percent of tracer bound to antibody, using the succinyl-digoxigenin–thyroside derivative.

Results

Results of the assay with SDT of patients' sera with various thyroxine concentrations are shown in Figure 1 (top). The samples have been grouped on the basis of thyroxine concentrations—low, medium, and high. The percentage bound for the low thyroxine group is significantly higher than that of the middle (P < 0.001) and that of the high (P < 0.05) group.

Figure 1 (bottom) shows the results for the same samples assayed under the same conditions with the TME derivative. There was no significant difference between the samples within a group or between groups.

We prepared standard curves from two serum samples that differed in percentage bound when assayed with the SDT derivative, to establish what differences in binding there would be between the two samples at various digoxin concentrations. Each serum sample was used to prepare two standard curves, one with use of the SDT derivative and one with use of the TME derivative. The curves, shown in Figure 2, illustrate that standard curves with quite different slopes resulted when the SDT was used, but there was little difference between the standard curves produced with the TME derivative.

The sera from the dogs dosed with thyrotropin showed a mean change from baseline of −1.9 (±3.7 SD) in percentage
bound when assayed with the SDT derivative. The change was +0.1 (±1.2) when assayed with the TME derivative. Significantly less variation occurred with the TME (P < 0.005). The data for one of the dogs are illustrated in Figure 3. Significant variation in percent bound is seen only when samples were assayed with use of the SDT derivative.

Discussion

Differences in the binding of the SDT tracer with different normal sera observed in this study are similar to those previously reported. It is unlikely that these discrepancies are related to lack of specificity of the antiserum used in the assay, especially since they are present only when the SDT derivative is used.

Nonspecific interference of serum components with the 125I-labeled digoxin radioimmunoassay appears to be eliminated with the use of the TME derivative in this assay. The slope as well as the height of the standard curve is related to the percent bound of the zero standard. Thus, determination of the percent of radiolabeled tracer bound to antibody in a serum sample with no digoxin present is both a convenient and a valuable tool in assessing the variation among standard curves prepared in different norma sera.

Inspection of the standard curves in Figure 2 (top) shows that if a sample of the serum from the curve with the open circles having a concentration of 1.0 ng/ml (1.0 µg/liter) were read from the standard curves with the closed circles, as could be the case in a clinical setting, the result would be an apparent concentration of 0.2 ng/ml. Conversely, if serum sample from the curve with closed circles having a concentration of 0 ng/ml were read from the curve with open circles, the concentration would be assumed to be 0.8 ng/ml. These errors do not occur when TME tracer is used (bottom), because the standard curves are essentially the same.

Our studies show that thyroxine concentration may be related to variation in binding of the SDT tracer in the digoxin radioimmunoassay. A possible explanation for this is that thyroxine is bound in the blood by specific proteins: thyroxine-binding globulin and thyroxine-binding prealbumin. These proteins may have a measurable effect on a derivative in which the radioactive iodine is bound to tyrosine, such as those used in the digoxin radioimmunoassay. If these derivatives bind to the proteins or if their binding is enhanced by them, an apparent increase in the percentage bound would result. Thyroxine-binding proteins are less saturated at low concentrations of thyroxine and, as a result, their capacity for influencing the tracer binding may be increased (8). At high serum thyroxine concentrations there appears to be less nonspecific binding of the tracer. This may be because the thyroxine-binding proteins are more nearly saturated.

Variations in thyroid status may partially explain between-sample variations in tracer binding in the digoxin assay. When the dogs were dosed with thyrotropin, there was an unmistakable influence on the binding of the SDT derivative. Occasional variations in binding of the SDT derivative before dosing with thyrotropin have been noted, but these appear to be random occurrences. Evidently thyroid variations are complex, and more than one factor may be responsible for variable tracer binding.

Our data indicate that results vary when the SDT derivative is used in this assay system. Between-sample variation with this derivative may be related to factors affecting the nonspecific binding of the derivative, such as thyroxine status. The data obtained with the TME derivative, however, suggest little change in binding between samples. Although it is not clear why the binding of the TME derivative was consistent, we may speculate that because of its molecular configuration or the presence of the digitoxose sugars, less nonspecific binding occurred. The use of the TME derivative in the system described eliminates much of the between-sample variation associated with the digoxin radioimmunoassay, and, as a result, errors in estimating digoxin concentrations will be decreased.

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References


