

The measurement of digitoxin in human serum by radioimmunoassay

George C. Oliver Jr., ... , Daniel L. Brasfield, Charles W. Parker

J Clin Invest. 1968;47(5):1035-1042. <https://doi.org/10.1172/JCI105793>.

Research Article

A sensitive, specific, and relatively simple immunoassay permitting measurement of pharmacological levels of digitoxin in human serum has been developed. The assay involves binding of ^{125}I -labeled tyrosine-digitoxigenin (specific activity > 400 mc/mg) by rabbit antibody to digitoxin. Antibody-bound radioactivity is precipitated by addition of a second antibody (goat anti-rabbit gamma globulin), and precipitate radioactivity is measured. Unlabeled digitoxin can be determined by the extent to which it competes with ^{125}I -labeled digitoxigenin and thus reduces precipitation of radioactivity. Before the assay, unlabeled digitoxin is extracted from serum with chloroform, and the chloroform solution is evaporated to dryness. Quantitation is accomplished by reference to a standard curve in which known amounts of digitoxin are added to normal serum. As little as 1 μg of digitoxin per ml of serum produces significant reduction in precipitate radioactivity.

The sera of 5 patients were analyzed before and after digitalization. A highly significant reduction in precipitate counts in the postdigitalization sera was observed ($P < 0.001$). Serum digitalis levels were measured in 19 patients receiving no digitalis and in 19 patients taking digitoxin or digitalis leaf. Little or no digitalis-like activity was detected in control sera, whereas serum levels averaged 27 $\mu\text{g}/\text{ml}$ in those on digitalis (range 4-60 $\mu\text{g}/\text{ml}$, $P < 0.001$). Patients judged clinically to show digitalis toxicity in general had higher levels than those [...]

Find the latest version:

<https://jci.me/105793/pdf>



The Measurement of Digitoxin in Human Serum by Radioimmunoassay

GEORGE C. OLIVER, JR., BRENT M. PARKER, DANIEL L. BRASFIELD, and CHARLES W. PARKER

From the Divisions of Cardiology and Immunology, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT A sensitive, specific, and relatively simple immunoassay permitting measurement of pharmacological levels of digitoxin in human serum has been developed. The assay involves binding of ^{125}I -labeled tyrosine-digitoxigenin (specific activity > 400 mc/mg) by rabbit antibody to digitoxin. Antibody-bound radioactivity is precipitated by addition of a second antibody (goat anti-rabbit gamma globulin), and precipitate radioactivity is measured. Unlabeled digitoxin can be determined by the extent to which it competes with ^{125}I -labeled digitoxigenin and thus reduces precipitation of radioactivity. Before the assay, unlabeled digitoxin is extracted from serum with chloroform, and the chloroform solution is evaporated to dryness. Quantitation is accomplished by reference to a standard curve in which known amounts of digitoxin are added to normal serum. As little as 1 μg of digitoxin per ml of serum produces significant reduction in precipitate radioactivity.

The sera of 5 patients were analyzed before and after digitalization. A highly significant reduction in precipitate counts in the postdigitalization sera was observed ($P < 0.001$). Serum digitalis levels were measured in 19 patients receiving no digitalis and in 19 patients taking digitoxin or digitalis

This work was presented at the 39th annual meeting of the Central Society for Clinical Investigation and abstracted in 1966 *J. Lab Clin. Med.* 68: 1002 and in 1967 *J. Clin. Invest.* 46: 1101.

Charles W. Parker is a recipient of a Research Career Development Award of the National Institute of Allergy and Infectious Disease.

Received for publication 16 August 1967 and in revised form 21 December 1967.

leaf. Little or no digitalis-like activity was detected in control sera, whereas serum levels averaged 27 $\mu\text{g}/\text{ml}$ in those on digitalis (range 4–60 $\mu\text{g}/\text{ml}$, $P < 0.001$). Patients judged clinically to show digitalis toxicity in general had higher levels than those without signs of toxicity. Patients receiving digoxin had little or no detectable digitalis in their serum with this method.

In addition to the assay itself, other potential uses of the antidigitalis antibody include treatment of digitalis toxicity and studies on the tissue localization of digitalis.

INTRODUCTION

Despite extensive use of digitalis for nearly two centuries, no method of measuring serum levels of the drug has been developed that is entirely satisfactory. Previously described methods have been either too cumbersome, too insensitive, or too nonspecific to be suitable for routine clinical use.

Stimulated by the success of radioimmunoassays in measuring minute levels of substances such as insulin and growth hormone, we have investigated the use of such a technique for digitalis assay. With this method, we have been able to develop a convenient means for the measurement of pharmacological levels of digitoxin in human sera.

METHODS

Paper chromatography

Unless otherwise stated, paper chromatography was performed in the following manner: Whatman No. 1 paper was impregnated with formamide by passing the paper through a solution of ethanol-formamide (1:1) and al-

lowing it to air-dry in a vertical position for 15–30 min. After materials were spotted, the paper was developed in the ascending manner with a solution of methyl ethyl ketone-xylene (1:1). When the chloroform-tetrahydrofuran-formamide system is referred to, the only change consists of replacing the methyl ethyl ketone-xylene solution with chloroform-tetrahydrofuran-formamide (100:100:13).

Digitalis products were located by the *m*-dinitrobenzene-KOH reaction (1) which indicates the presence of an intact butenolide ring. The tyrosine group was located by the 1-nitroso-2-naphthol reaction (2).

Synthesis of 3-*O*-succinyl digitoxigenin (SDG)

The method of Yamada was used (3). 860 mg of succinic anhydride¹ and 442 mg of digitoxigenin² were dissolved in 13.4 ml of pyridine. The solution was protected from light and allowed to react at room temperature for 3 months. The solution was poured into 75 ml of cold 2 N H₂SO₄. The solid product was isolated by filtration and washed with cold water. It was then dissolved in 150 ml of chloroform-methanol (2:1). The chloroform-methanol solution was washed once with 25 ml of 1 N H₂SO₄, and three times with water. 25 ml of methanol was added after each washing. The organic phase was dried over anhydrous sodium sulfate and taken to dryness on a rotary evaporator. The residue was dissolved in 15 ml of hot ethanol, and hot water was added to turbidity. The solution was allowed to cool to room temperature and then left at 4°C for 48 hr. The resultant crystals were isolated by filtration and washed three times with cold ethanol-water (3:2). The final product was a white powder, with melting point of 220–227°C. The yield varied from 60–75%.

Preparation of antigen

SDG was coupled by two different methods to human serum albumin and bovine serum albumin.

Carbodiimide method. 50 mg of human serum albumin³ was dissolved in 25 ml of water (pH 5.5). The solution was filtered, and 30 mg of EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride]⁴ was added. 20 mg of SDG dissolved in 2 ml of dimethylformamide was then added dropwise while stirring, and the pH was adjusted to 5.5 with dilute HCl or NaOH. After 10 min, an additional 10 mg of EDC was added. The solution was stirred at room temperature for 18 hr. The reaction mixture was then dialyzed exhaustively (1 wk) against water with frequent changes of the outside solution. The suspension was cleared by centrifugation, and the soluble protein fraction was lyophilized. The product contained at least five digitoxigenin molecules per molecule of protein [*m*-dinitrobenzene assay (4)].

Isobutylchloroformate method. A modification of the method described by Erlanger, Borek, Beiser, and Lieber-

man (5) was used. 0.15 ml of tributylamine and 280 mg of SDG were added to 5.85 ml of dioxane. After the solution had been cooled to 10°C, 0.08 ml of isobutylchloroformate⁵ was added. The solution was stirred for 20 min with gradual cooling to 4°C. The mixture was then added rapidly to a well-stirred and cooled solution of 840 mg of bovine serum albumin³ in a mixture of 22.0 ml of water, 22.0 ml of dioxane, and 0.84 ml of 1 N NaOH. After 1 hr another 0.4 ml of 1 N NaOH was added. The solution was stirred at 4°C overnight. The product was purified by dialysis as described above. It contained at least 13 molecules of digitoxigenin per molecule of protein (*m*-dinitrobenzene assay). The synthetic steps are summarized in Fig. 1

Synthesis of 3-*O*-succinyl digitoxigenin tyrosine methyl ester (SDG-TME)

100 mg of SDG was dissolved in 2.14 ml of 0.255% (v/v) mixture of tributylamine in dioxane. The SDG solution was cooled to 10°C, and 0.03 ml of isobutylchloroformate was added. The solution was stirred for 20 min, whereupon a cooled solution of tyrosine methyl ester in dioxane-H₂O was added rapidly. The latter contained 49 mg of tyrosine methyl ester hydrochloride dissolved in a mixture of 7.85 ml of water, 7.85 ml of dioxane, and 0.3 ml of 1 N NaOH. Stirring was continued at 10°C for 1 hr after which 0.14 ml of 1 N NaOH was added. The solution was stirred overnight in an ice bath that gradually reached room temperature. The following morning the solution was cooled to 4°C, and 185 ml of cold water was added. The precipitate that formed was isolated by centrifugation and washed four times with 30 ml of water, 30 ml of 1 N HCl, 40 ml of 1 M NaHCO₃, and 40 ml of water (yield 50%, melting point 99°C). By paper chromatography (methyl ethyl ketone-xylene and chloroform-tetrahydrofuran-formamide systems) the product gave a single dinitrobenzene positive, 1-nitroso-2-naphthol positive spot. Chemical analysis⁶ for C₃₇H₄₆N₁O₉; calculated: C 68.18, H 7.58, N 2.15; found C 66.75, H 7.6, N 2.2. The structural formula is given in Fig. 2.

Preparation of radioactive hapten (SDG-TME-I)*

A radioactive hapten was prepared by iodinating SDG-TME by a modification of the method of Hunter and Greenwood (6). 30 μl of a 10 μg/ml solution of SDG-TME was mixed with 80 μl of 0.5 M phosphate buffer (pH 7.4). Approximately 20 μl of carrier-free ¹²⁵I (as Na¹²⁵I in NaOH, representing 2 mc) was added, and, exactly 2 min later, 20 μl of a freshly prepared aqueous solution of chloramine-T (7 mg/ml) was added. 2 min later, 20 μl of a freshly prepared aqueous solution of sodium metabisulfite (7 mg/ml) was added. After an additional 2 min, 0.5 ml of dimethylformamide and 0.3 ml

¹ Eastman Kodak Co., Rochester, N. Y.

² Aldrich Chemical Co., Inc., Milwaukee, Wis.

³ Pentex, Inc., Kankakee, Ill.

⁴ Ott Chemical Co., Muskegon, Mich.

⁵ K & K Laboratories, Inc., Plainview, N. Y.

⁶ Schwarzkopf Microanalytical Laboratories Inc., Woodside, N. Y.

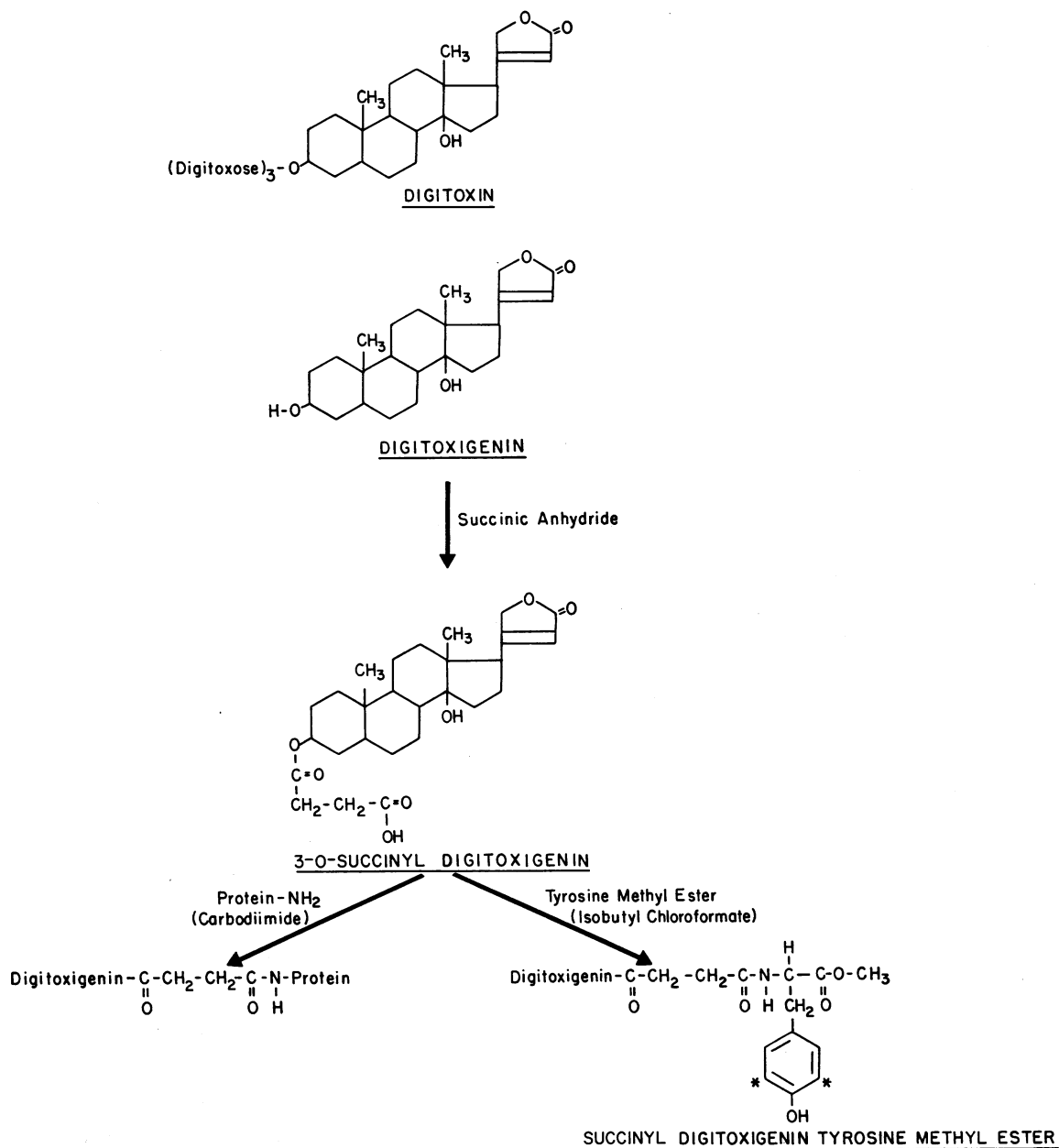


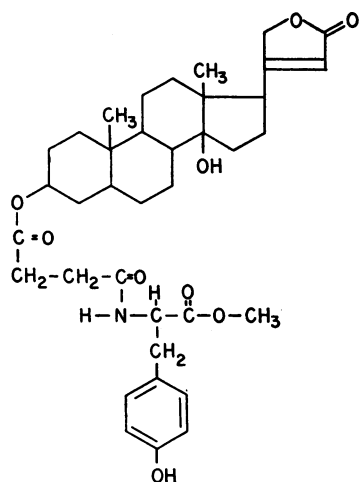
FIGURE 1 The structural formulas of the digitoxin and the aglycone, digitoxigenin, are shown. The synthetic steps leading to antigen (digitoxigenin-protein complex) and ligand are shown. See Methods section for further details. The asterisks indicate the probable position of the radioactive iodine molecule after iodination.

of H₂O were added, and the product was immediately purified by serial transfer through seven tubes containing a small amount (80–100 mg) of Amberlite IRA 400, chloride form, (Rohm & Haas Co., Philadelphia, Pa.). The final product was 96% pure by chromatography (methyl ethyl ketone-xylene) and had a minimum specific activity of > 400 mc/mg. In equilibrium dialysis experiments, as much as 98% of the counts could be bound by

rabbit antidigitoxigenin antibody, and there was very little nonspecific binding to nonimmune rabbit gamma globulin (< 1%).

Immunological procedures

Randomly bred rabbits were immunized with 3.2 mg of human serum albumin (or bovine serum albumin)-SDG complex in complete Freund's adjuvant (0.4 ml/per foot-



3-O-Succinyl Digitoxigenin Tyrosine Methyl Ester

FIGURE 2 The structural formula of the succinyl tyrosine derivative of digitoxigenin is shown.

pad). Animals were boosted with 1 mg of antigen in complete adjuvant at 1 month and every few months thereafter. Sera were obtained by cardiac puncture 5-7 days after booster injections. "Normal" rabbit gamma globulin and rabbit gamma globulin containing antidigitoxigenin antibody were purified by precipitation with ammonium sulfate at 50% of saturation followed by diethylaminoethyl (DEAE) chromatography. The DEAE cellulose and the globulin fractions were equilibrated with 0.02 M phosphate, pH 7.0. The initial protein fractions eluted from the column were pooled on the basis of 280 $m\mu$ of absorbance. Gamma globulin concentrations were based on 280 $m\mu$ of absorbance, and 1.5 was used as the value for a 1 mg/ml solution (1 cm light path).

Goat antibody to rabbit gamma globulin was obtained as follows: 50 mg of rabbit gamma globulin³ in 10 ml of complete Freund's adjuvant was injected subcutaneously into multiple sites on the back and thighs of the goat. The animal was bled at 3 wk and at intervals thereafter. A crude globulin fraction was obtained by precipitation with ammonium sulfate at 20% of saturation and labeled "goat anti-rabbit gamma globulin."

Collection of patient blood samples

Venous blood was collected in glass tubes containing no anticoagulant and allowed to clot at room temperature. Serum was separated by centrifugation at 4°C and immediately analyzed or frozen at -20°C for further analysis. Repeated analysis of samples showed no loss of digitalis activity after as long as 3 months of storage.

Extraction of digitalis from serum

As will be discussed later, serum could not be analyzed directly in the assay system. Digitalis was extracted from serum and an analysis performed on the extract. 5 ml of serum was pipetted into a 15 ml glass stoppered centri-

fuge tube, and 5 ml of reagent grade chloroform was added. The tubes were gently inverted 10 times and allowed to settle for 10 min. The inversion and settling process was repeated twice. After the third series of inversions, the tubes were centrifuged for 30 min in a refrigerated centrifuge (4°C, 1200 *g*). The upper serum layer and a milky precipitate layer at the interface were removed by aspiration, and the chloroform layer was filtered. 1 ml aliquots of the filtrate were transferred to each of the two assay tubes and evaporated to dryness in a water bath (<90°C). The assay was subsequently carried out in these tubes. The efficiency of the extraction was tested by adding tritiated digitoxin⁷ to serum and counting the chloroform extract in a liquid scintillation counter.⁸ The efficiency of extraction was 53.6 ± 5.6%.

Assay procedure

Preliminary experiments were run to determine the dilution of rabbit antibody that resulted in the precipitation of approximately 50% of the radioactive hapten in the presence of excess goat anti-rabbit gamma globulin sera (see below). 20 μ l of a mixture of the appropriate amount of rabbit antibody to digitoxigenin (usually a dilution of about 1:300) and carrier rabbit gamma globulin (adjusted to give a total protein concentration of 0.5 mg/ml) were added to each assay tube. 50 μ l of freshly centrifuged radioactive hapten (SDG-TME-I*) in 2.5% egg albumin was next added. The radioactive hapten was diluted so that 50 μ l yielded approximately 10,000 cpm.⁹ Phosphate saline buffer (0.15 M NaCl, 0.008 M K₂PO₄, 0.002 M NaH₂PO₄, pH 7.4) was added to bring the volume of each tube to 200 μ l. The tubes were mixed well and allowed to incubate for 1 hr at room temperature, after which they were left at 4°C overnight. The following morning 200 μ l of goat anti-rabbit gamma globulin (in the region of antibody excess) was added to each tube. After the tubes were mixed, they were allowed to incubate for 1 hr at room temperature, during which time total counts were measured. They were then incubated at 4°C for 4 hr and centrifuged at 4°C (1000 *g*) for 30 min. The supernatant solution was removed by careful aspiration. The precipitate was washed once with 1 ml of cold phosphate saline, and counts were then measured. All samples were run in duplicate, and the per cent counts in the precipitate were calculated for each tube.

Precision and reproducibility

The precision of the method was tested by an analysis of the per cent counts in the precipitate of 51 duplicate tubes. The coefficient of variation was ±6.7%.

A similar analysis was performed on repeated measure-

⁷ New England Nuclear Corp., Boston, Mass.

⁸ Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3375, Packard Instrument Co., Inc., Downers Grove, Ill.

⁹ Packard Model 410A autogamma spectrophotometer, well-type, Packard Instrument Co., Inc., Downers Grove, Ill.

ment of serum digitoxin levels. A total of 29 measurements was made on the serum from 10 patients. For serum levels in the range 0–10 $\mu\text{g}/\text{ml}$, the coefficient of variation was $\pm 31.7\%$; in the range 10–20 $\mu\text{g}/\text{ml}$, the coefficient of variation was $\pm 19\%$; and in the range 20–45 $\mu\text{g}/\text{ml}$, coefficient of variation was 11%

RESULTS

Several different techniques were used to demonstrate the presence of rabbit antibody to digitoxin. First, positive interfacial precipitation tests were obtained with rabbit anti-SDG–human serum albumin (HSA) as the antibody and SDG–bovine serum albumin (BSA) as the antigen. This result, while not conclusive, suggests the presence of rabbit antibody. Secondly, in equilibrium dialysis experiments when SDG-TME-I* as hapten was used, as much as 98% of the radioactivity could be bound to the antibody with essentially no non-specific binding ($< 1\%$) to “nonspecific” rabbit gamma globulin. Finally, the radioimmunoassay itself gave clear evidence that antidigitoxin antibody had been produced. The precipitation of radioactivity was markedly increased in the presence of specific antibody as compared with “nonspecific” gamma globulin. As little as 1–2 $\text{m}\mu\text{g}$ of digitoxin resulted in easily detectable reduction in the specific precipitation of radioactivity (Fig. 3). The limited amount of nonspecific precipitation of radioactivity that occurred in the presence of “nonspecific” gamma globulin was not inhibited by digitoxin.

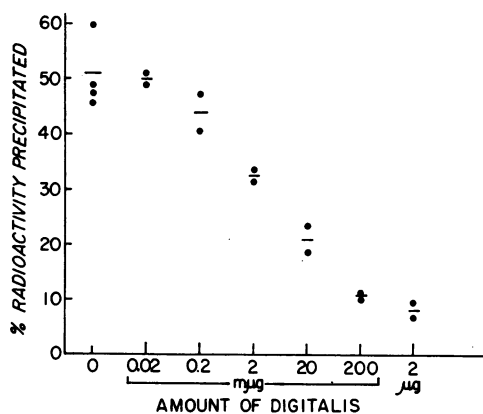


FIGURE 3 The results of a typical calibration curve are shown. In this assay, as little as 0.2 $\text{m}\mu\text{g}$ of digitoxin per assay tube produced detectable reduction in precipitate radioactivity. In the range of 0.2–200 $\text{m}\mu\text{g}$, when results are plotted on a semilogarithmic scale (as is shown), the curve is linear.

Digitalis in human serum could be measured by its ability to inhibit the precipitation of SDG-TME-I* in the double antibody assay. Chloroform extracts of human serum containing no digitalis caused about a 10% reduction in precipitate counts. Therefore, in establishing calibration curves, known amounts of digitalis were added to “normal” human serum and extracted with chloroform. The sera of 19 persons receiving digitoxin or digitalis leaf have been analyzed. In every instance significant inhibition of precipitation over control sera was observed ($P < 0.001$). The serum levels of digitoxin as judged by this technique were in the range of 4–60 $\text{m}\mu\text{g}/\text{ml}$ (Fig. 4). The sera of five patients were analyzed before and after digitalization. In each instance marked inhibition was found after digitalization [$P < 0.001$ (Fig. 5)].

In contrast to digitoxin and digitalis leaf, sera of patients taking digoxin showed in general very little inhibition. This finding is not due entirely to the lower levels of digoxin found in the sera of patients on digoxin (7) but is due at least in part to a reduced ability of digoxin to compete for antibody sites. Digoxin in amounts of 5–12 $\text{m}\mu\text{g}$ in saline caused little inhibition, and in general in our assay system, on an equimolar basis, digoxin was about one tenth as active as digitoxin (Table I).

TABLE I
Per Cent of Inhibition

Amount	Digitoxin	Digoxin
$\text{m}\mu\text{g}$		
2	21.7	—
5	36.0	9.7
10	53.2	18.0
25	66.0	31.6
50	79.8	40.1

Comparison between digitoxin and digoxin. For a given degree of inhibition of precipitate radioactivity, roughly ten times more digoxin than digitoxin is needed.

The results of analysis of 19 “normal” sera indicated that nonspecific serum inhibition did not vary to any major extent from individual to individual. Efforts to demonstrate that the serum inhibitor is a circulating steroid have been inconclusive to date. Cholesterol caused moderate inhibition at a level of 150 μg , but hyperlipemic sera did not exhibit more inhibition than normal sera.

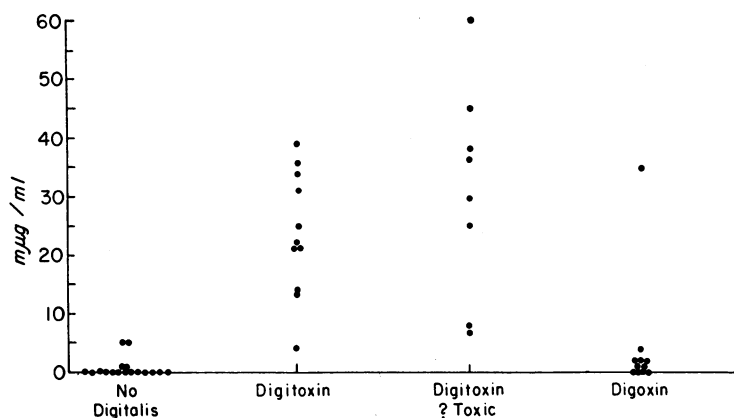


FIGURE 4 Serum digitalis levels in controls (on no digitalis), patients maintained on digitoxin or digitalis leaf, and patients maintained on digoxin. In addition, data are presented on patients suspected clinically of showing signs of digitalis toxicity (? Toxic). With only two exceptions, essentially no digitalis-like activity was detected in the sera of control patients, and, in these two cases, the amount was small. In contrast, every patient on maintenance digitoxin or digitalis had detectable levels. In general, those patients who were suspected of having digitalis toxicity had higher levels than those not toxic, but overlap was present. See Results section for further discussion. Patients on digoxin had in general very low serum levels. The one exception was a patient who had been acutely digitalized with digoxin.

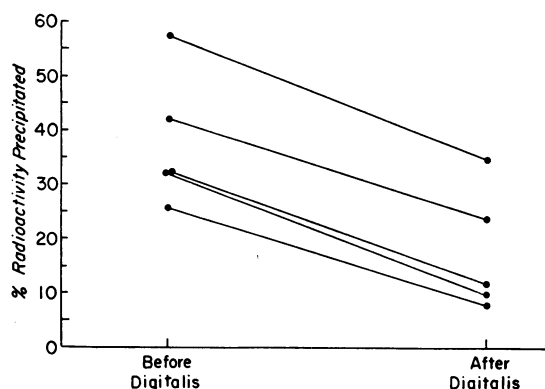


FIGURE 5 Results from five patients before and after digitalization with digitoxin. Control and postdigitalization sera were compared in the same immunoassay. Control studies indicated that prolonged storage of digitalis in the presence of serum at -20°C did not diminish its inhibitory activity in the immunoassay. In every case, postdigitalization sera caused more inhibition of precipitate radioactivity than predigitalization sera ($P < 0.001$). The variation in predigitalization serum values shown in the graph (from 25 to 60% precipitation of radioactivity) reflects the fact that the immunoassays were carried out at different times under somewhat different conditions. In each instance, other normal sera evaluated at the same time gave comparable values. Despite some variation in control serum values from assay to assay repeated analysis of digitalis-containing sera gave reproducible results.

Physiological levels of hydrocortisone, androsterone, and dehydroepiandrosterone produced little, if any, inhibition. Progesterone and testosterone in large amounts (500 $\text{m}\mu\text{g}$) caused moderate inhibition.

Sera from five patients who were receiving digitalis currently or who had taken digitalis in the past have been screened for antidigitoxin antibodies. Such antibodies have been found in a patient who developed thrombocytopenia (8). Undiluted sera were incubated with SDG-TME-I* for 1 hr at 37°C , and proteins were separated by electrophoresis (oxid strips). The strips were then cut into 1 cm sections and counted in a gamma counter. Radioactivity was found at the origin and with the albumin fraction. In no instance was radioactivity found in the gamma globulin band. Thus there was no evidence for the presence of antidigitoxin antibodies. Studies on a more extended series of sera are in progress.

DISCUSSION

The radioimmunoassay described in this paper provides a simple, sensitive, and specific means of measurement of serum digitoxin levels in man.

Patients receiving therapeutic doses of digitoxin usually had serum levels in the 5–40 m μ g/ml range by radioimmunoassay. This finding confirms the results of Lukas and Peterson (9) who found levels of 10–50 m μ g/ml by an independent method. In general, the highest serum levels of digitoxin were in patients who were suspected of having digitalis toxicity clinically, a fact which supports the data of Doherty and Perkins (10) who found a significant correlation between blood and myocardial digitalis levels in dogs. We suspect that the relationship between blood levels and digitalis toxicity may be better than that shown in Fig. 4. In the two patients who had the lowest serum levels of digitalis there was some uncertainty as to the clinical diagnosis of toxicity.

The radioimmunoassay discussed herein appears to possess significant advantages over other methods for measuring serum levels of digitalis. Bioassay methods such as those with the duck embryo heart are too laborious for routine use (11). The rubidium uptake technique of Lowenstein (7) lacks specificity. In his first publication (7) he noted considerable overlap between patients on digitalis and those not, and a number of false positive tests occurred. In a subsequent publication, Lowenstein and Corrill (12) stated they had eliminated the false positives by extracting plasma with methylene dichloride. This technique appeared to improve the sensitivity of their assay, but they presented no new data on the overlap problem. Lukas and Peterson (9) have recently described a double isotope assay for digitoxin that appears to be quite sensitive and specific. However, this technique is lengthy and laborious and is unsuitable for routine clinical use. The studies of Okita, Talso, Curry, Smith, and Geiling (13) and Doherty and Perkins (14) in which radioactive digitalis preparations were administered to human patients have added to our knowledge of the metabolism of digitalis preparations. However, these studies can be used only for acute studies and are not useful for measuring serum levels in a problem patient.

At present we can measure serum levels of digitoxin in patients receiving digitoxin or digitalis leaf. The specificity of the radioimmunoassay is such that digoxin, which differs from digitoxin only in the presence of a hydroxyl group at the

C-12 position, is bound only about one tenth as well as digitoxin. In view of the marked structural similarity between digoxin and digitoxin the substantial reduction in digoxin binding implies that there is a tight fit between digitoxin and antibody at this position on the steroid ring. It is of interest that Butler and Chen (15) using antidigoxin antibody found that binding of the homologous ligand, digoxin, was favored over that of digitoxin by about the same order of magnitude. The extent to which other structural analogues of digitoxin would cross-react immunologically is not known at present. On purely structural grounds one would predict that any digitalis preparation containing the digitoxigenin moiety would be bound well by the anti-succinyl digitoxigenin antibody. Because of the relatively weak binding of digoxin and the low serum levels of this drug, serum digoxin levels cannot be measured with the current immunoassay system. Work is in progress to prepare antidigoxin antibodies and ¹²⁵I-labeled digoxin so that the radioimmunoassay can be extended to patients receiving digoxin.

With the present assay system, results are not obtained before 36 hr. Work in progress indicates that it should be possible to shorten the time of the assay to 4 or 5 hr permitting the measurement of levels of digitalis in serum in acute therapeutic situations. Encouraging results have been obtained with ammonium sulfate at 33% of saturation to separate antibody-bound and free SDG-TME-I*. The extent of nonspecific precipitation of radioactivity with ammonium sulfate is relatively high, but with recent modifications nonspecific binding has been reduced to 10% of the total radioactive hapten present.

As previously mentioned, in a parallel independent study Butler and Chen (15) recently have prepared rabbit antidigoxin antibodies. Their method of immunization was similar in principle to our own in that the immunizing antigen was a conjugate of digitalis with protein. Their conjugation procedure involved the oxidation of digoxin with sodium periodate followed by conjugation to protein. They demonstrated the presence of antibody by equilibrium dialysis with ³H-labeled digoxin as the ligand. However, they have not been able to measure serum levels of digitalis, and it appears that the sensitivity of their system would

have to be increased by at least two to three orders of magnitude in order for this to be possible. The much greater sensitivity of the radioimmunoassay described in this paper is due at least in part to the use of a labeled digitalis molecule with high specific activity. Additional factors may include the relative affinities of the antidigitalis antibodies involved and the precise nature of the immunoassay procedure.

The chloroform extraction step in the radioimmunoassay is necessary in order to reduce non-specific interference with the assay by normal serum. Conceivably antidigitalis antibodies present in the serum of patients receiving the drug might interfere with the extraction. However, preliminary data have failed to reveal significant levels of the antibody. During the extraction, steroids and other nonpolar substances normally present in serum enter the organic phase. Cholesterol, testosterone, and progesterone are capable of inhibiting the precipitation of radioactivity in the immunoassay, but high concentrations are required. At present, there is no indication that physiological variations in the serum levels of these substances would influence the immunoassay to any major extent. However, the possibility remains that high hormonal levels achieved pharmacologically might exert a digitalis-like effect in the assay system.

Other uses of the antidigitalis antibody are possible. For instance, it is possible that antidigitalis antibody could be administered to patients with digitalis intoxication to reverse the toxic process by combining with serum or tissue digitalis. We are currently investigating this possibility. Another potential use for the antibody would be to help ascertain the location of digitalis in heart muscle with fluorescein or ferritin labeling. We believe, however, that the main usefulness of the antibody will be its ability in the assay to afford a relatively rapid assessment of digitalis levels and hence of the need for more or less drug.

ACKNOWLEDGMENTS

We wish to thank Barbara Bloss and Shirley Godt for their valuable technical assistance.

This work was supported by grants 1R01 HE 11233-01, HE-11034, AI 04646, and AI 00219 of the U. S. Public

Health Service. Dr. Brasfield was supported by Student Research Training Grant T5-GM 1608-10.

REFERENCES

1. Jung, Z. 1963. Steroid glycosides and their aglycones. *In* Paper Chromatography. I. M. Hais and K. Macek, editors. Academic Press Inc., New York. 402.
2. Hais, I. M. 1963. Amino acids. *In* Paper Chromatography. I. M. Hais and K. Macek, editors. Academic Press Inc., New York. 449.
3. Yamada, A. 1960. Synthesis of succinoyl and phthaloyl derivatives of digitoxigenin, gitoxigenin and oleandri-genin. *Chem. Abstr.* **54**: 6807.
4. Wright, S. E. 1960. The Metabolism of Cardiac Glycosides. Charles C Thomas, Springfield, Ill. 22.
5. Erlanger, B. F., F. Borek, S. M. Beiser, and S. Lieberman. 1957. Steroid-protein conjugates. I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortisone. *J. Biol. Chem.* **228**: 713.
6. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of Iodine-131 labeled human growth hormone of high specific activity. *Nature.* **194**: 495.
7. Lowenstein, J. M. 1965. A method for measuring plasma levels of digitalis glycosides. *Circulation.* **31**: 228.
8. Young, R. C., R. L. Nachman, and H. I. Horowitz. 1966. Thrombocytopenia due to digitoxin. *Am. J. Med.* **41**: 605.
9. Lukas, D. S., and R. E. Peterson. 1966. Double isotope dilution derivative assay of digitoxin in plasma, urine, and stool of patients maintained on the drug. *J. Clin. Invest.* **45**: 782.
10. Doherty, J. E., W. H. Perkins. 1966. Tissue concentration and turnover of tritiated digoxin in dogs. *Am. J. Cardiol.* **17**: 47.
11. Friedman, M., and R. Bine, Jr. 1947. Employment of the embryonic duck heart for the detection of minute amounts of a digitalis glycoside (lanatoside C). *Proc. Soc. Exptl. Biol. Med.* **64**: 162.
12. Lowenstein, J. M., and E. M. Corrill. 1966. An improved method for measuring plasma and tissue concentrations of digitalis glycosides. *J. Lab. Clin. Med.* **67**: 1048.
13. Okita, G. T., P. J. Talso, J. H. Curry, Jr., F. D. Smith, Jr., and E. M. K. Geiling. 1955. Metabolic fate of radioactive digitoxin in human subjects. *J. Pharmacol. Exptl. Therap.* **115**: 371.
14. Doherty, J. E., and W. H. Perkins. 1962. Studies with tritiated digoxin in human subjects after intravenous administration. *Am. Heart J.* **63**: 528.
15. Butler, V. P., Jr., and J. P. Chen. 1967. Digoxin-specific antibodies. *Proc. Natl. Acad. Sci. U. S.* **57**: 71.