Radioimmunoassay: Generally and of Digoxin Specifically

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This presentation is intended to review the general principles of radioimmunoassay and to use as a model the radioimmunoassay of digoxin with detailed discussion (*1*). A brief version of the digoxin assay will be given first as a foundation for a more detailed discussion of radioimmunoassay in general. Finally, digoxin assay in detail and a presentation of the practical experience with this in a general hospital setting will be covered.

Radioimmunoassay: Fundamentals

The term radioimmunoassay (RIA) is applied to an analytical technique employing competitive binding assay. The "radio" refers to the fact that the technique uses radioactivity, with instrumental quantitation based on radioactivity measurement by counting. This feature affords the very high sensitivity that is characteristic of the method and makes it applicable to the measurement of extremely low concentrations of substances. Such low concentrations occur in patients because these substances have exceedingly high biological activity (e.g., hormones, enzymes, some drugs). In Table 1, representative ranges of sensitivity are tabulated for several of the common clinical laboratory assay methods. It is apparent that RIA far exceeds the sensitivity of the other methods and is indeed comparable to bioassay techniques. The nanogram weight unit commonly used is the same as the older "millimicrogram" or 10^{-9} gm.

Table 1. Assay Sensitivities With Different Techniques

Colorimetric	10 → 100 μg/ml
Ultraviolet absorption	1 → 10 μg/ml
Fluorometric	0.1 → 1 μg/ml
	or 100 → 1,000 μg/ml
Radioimmunoassay	0.2 → 10 μg/ml

Table 2. Calculation to Show Need for Analytical Sensitivity at ng/ml Level for Serum Digoxin Assay

Dose 0.5 mg = 500 mg
= 500,000 ng

Assuming: Distribution to total body
No excretion of drug

$$\frac{500{,}000}{50 \text{ kg} \times 1{,}000 \text{ ml/kg}} = 10 \text{ ng/ml}$$

Digoxin exemplifies a highly active drug where the daily dose is only a fraction of 1 mg for an average adult patient. In Table 2 a slightly oversimplified calculation illustrates why it is necessary to measure at the nanogram per milliliter concentration when the daily dose of the drug is only half a milligram.

The "immuno" part of radioimmunoassay refers to the common use of an immune protein employed in the method and responsible for its high degree of specificity. This specific protein may be a naturally occurring binding protein such as transferrin, transcortin, thyroxin binding globulin, or the intrinsic factor which mediates the gastrointestinal absorption of vitamin B_{12}. In other instances the specific protein may be a naturally occurring enzyme. Quite commonly the specific binding protein is a manufactured entity in the form of an antibody or antiserum deliberately produced biosynthetically in some animal and then isolated for use in the assay. These proteins are characterized by a high degree of specificity and strong binding affinity for their respective target chemical compounds. By virtue of these attributes reactions can occur at extremely low concentrations permitting the same high order of sensitivity as is afforded by the radionuclide used in the method.

The basic reaction employed in RIA is shown in Fig. 1. The unlabeled *ligand* is commonly that compound which we wish to measure and may be a small molecule or even an ion. The term ligand derives from the fact that this chemical entity is the one which binds to the protein *binding agent.* The binding agent shown in the center of the figure

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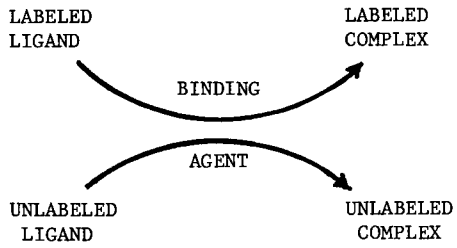

FIG. 1. Schematic diagram of competitive reactions involved in radiommunoassay.

is the "immuno" part of the assay and is the protein antiserum or other selective binding agent. The *labeled ligand* is the radioactive form of the ligand which is introduced in vitro under controlled conditions so that it competes with the unknown quantity of unlabeled ligand for a limited quantity of binding agent. By virtue of the radioactivity it is possible to measure the quantity of labeled ligand that exists in both the unbound and in the complexed or bound form. These proportions reflect the quantity of unlabeled ligand that must have been in the system at the time of the reaction. In the case of digoxin assay, the unlabeled ligand is the patient's serum digoxin level, the labeled ligand is the radioactive digoxin and the binding agent is a protein antibody to digoxin.

The four requirements essential to the conduct of any RIA are given in Table 3. First, it is essential to have a suitable binding agent, and not all "binding agents" are satisfactory for RIA. The specific protein must have a very high *affinity* for its ligand, the material to be measured. The possible sensitivity of the assay is proportional to the avidity with which the binding protein reacts with the ligand. A second attribute of the binding agent for effective RIA is a high degree of *specificity* for the ligand to be measured. This means that the binding protein must show great *selectivity* with respect to the ligand and be free from cross reactivity with related molecules. In the case of digoxin the available antibodies are sufficiently specific that they show no measurable cross reaction with the normally occurring steroid hormones which are chemically very similar to digoxin. In fact, good digoxin antibody reacts with digoxin 30 times more strongly than it does with the very closely related cardiac glycoside digitoxin.

The second requirement for RIA is the availability of a radioactive labeled form of the ligand to be measured. Most commonly this is chemically identical, but one may use a chemical derivative to incorporate the radioactivity while preserving reactivity with the binding agent. This labeled ligand must be extremely pure and of high specific activity with respect to the radioactive label. In general, the sensitivity of the assay will be proportional to the specific activity of the labeled ligand.

The third requirement is to have available some pure and assayed unlabeled ligand material to serve as the standard material for the calibration of the test. In the case of digoxin RIA, crystalline digoxin of very high chemical purity is available, and appropriate standard solutions can be prepared gravimetrically.

A fourth requirement for RIA is the availability of a separation technique which permits a reasonably simple separation of bound ligand (labeled and unlabeled) from the free or unbound ligand in the samples under analysis.

With this elementary background it is appropriate to return to our digoxin RIA model to look at the practical aspects of this assay. In Figure 2 a sample is shown in a single test tube in which all the steps of the method take place up to the final radioactivity counting. This example shows a sample containing 4 units of digoxin; these could be molecules, nanograms, or any kind of units of digoxin which are shown here in 1-2 ml of buffer.

The first step involves the addition of a known and constant quantity of radioactive ligand (digoxin or derivative) to each tube in the series. The

Table 3. Requirements for Radioimmunoassay

1. Binding agent
   - specific protein
   - antibody
   - enzyme
2. Radioactive ligand
3. Standard ligand
4. Separation technique

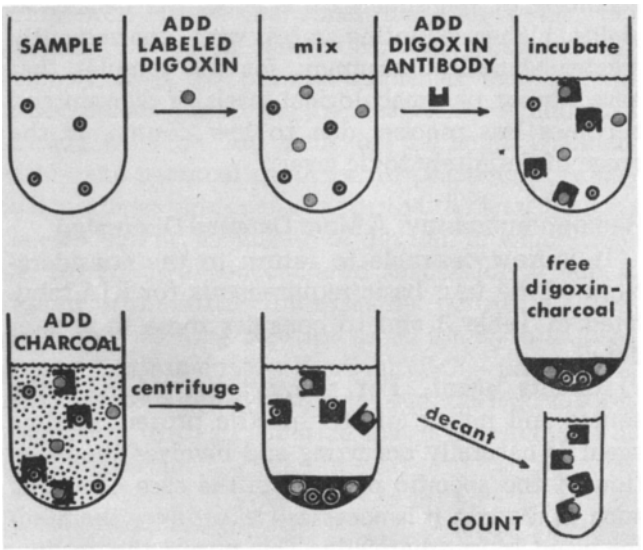

FIG. 2. One technique for digoxin radioimmunoassay.

quantity added is of the same general magnitude as the quantity of digoxin to be expected in the sample. In the example here, a similar 4 units of labeled digoxin, represented by the open circles, have been added.

The next step is to add the binding agent, digoxin antiserum. The amount of this has been selected to be only sufficient to bind approximately half of the amount of the labeled digoxin that was added in the previous step. The quantity of binding agent is constant and limited for all tubes in the series. At this point the tubes are set aside to incubate at room temperature for approximately 30 min. This allows the binding agent to find and couple to the digoxin molecules until all of the binding agent is saturated.

The next step is the separation of the free unreacted from the bound digoxin. This is accomplished by the addition of a controlled quantity, but an excess, of charcoal to absorb the digoxin, radioactive and plain, which did not find antibody molecules to which to bind. The antibody-digoxin complexes do not bind to the charcoal. The charcoal is separated from the mixture by centrifugation leaving the bound digoxin in the supernate. The final step is pouring off the supernate into a test tube for radioactive counting.

It now should be apparent that the test permits a known amount of radioactive digoxin to compete with an unknown amount of sample digoxin for a limited and fixed quantity of binding agent. The more digoxin is present in a patient's serum, the less radioactive digoxin can find an antibody to which to bind and carry through to be counted. In the final counting sample, low radioactivity counts correlate with a high patient digoxin level. It actually is possible to count the radioactivity in either the supernate or the residual charcoal fraction. Counting of the supernate is preferred because it yields higher counting rates with consequently greater statistical accuracy for the samples that have low or pharmacological levels of digoxin and becomes less precise, due to low counts, in the range of definitely toxic levels.

Radioimmunoassay: A More Detailed Discussion

It is now desirable to return to the considerations of the four basic requirements for RIA tabulated in Table 3 and to consider these in greater detail.

Binding agent. For many types of RIA the source and nature of the specific protein binding agent is naturally occurring and involves the isolation of the specific protein. In the case of a drug such as digoxin it is necessary to produce the binding agent biosynthetically. This means the production of antibodies to the compound and, as in the case of digoxin, involves first making the compound into an antigenic derivative. Smith, Butler, and Haber (2) accomplished this in the case of digoxin by the periodic oxidation of the terminal sugar moiety of the glycoside and the attachment of this by covalent chemical bonds to the amino group of a lysine moiety on human serum albumin. This produces a chemically stable protein conjugate which is antigenic in species other than man. The desired antidigoxin antibody is then produced by the immunization of rabbits over the course of 20-100 weeks to produce a high titre antiserum. This antiserum has a strong affinity for the ligand and a very high order of specificity for digoxin. The specificity is such that there is no measurable reaction between the antibody and levels of digoxin-like steroid hormones as high as 100 times those encountered under physiologic, pathologic, or even usual pharmacologic conditions. This high specificity of the antibody makes the test specific.

Solutions of the antibody are unstable but can be effectively preserved by lyophilization and/or freezing. Further protection of the antibody is afforded by the incorporation of some protective protein such as human serum albumin in the antibody solutions. The actual mass of antibody present in the working solutions used in the test is so small that simple adsorption to the glass walls of the bottles and tubes is a serious problem and requires that all containers be coated with silicone or be made of unreactive plastic. Working solutions of the antibody can be used for approximately 4 weeks if stored under refrigeration. Repeated freezing and thawing should be avoided since it inactivates the antibody.

Radioactive ligand. The second requirement for RIA is the availability of a suitable labeled ligand, a radioactive form of the substance to be measured. To achieve the high sensitivity necessary to assay very low concentrations, it is necessary to have a high specific activity in the labeled ligand. For most purposes a radiocarbon label cannot provide the desired specific activity. Tritium (^{3}H), is frequently used because even singly labeled molecules can have a specific activity a thousand times that of ^{14}C labeling. Furthermore, molecules can frequently be labeled with several radioactive hydrogen atoms. Tritium labeling is commonly easier and less expensive than radiocarbon synthesis. Tritium, however, is a pure beta emitter so that assay of the radioactivity usually has to be done by liquid scintillation counting, a technique requiring expensive equipment which is less commonly available than that for gamma counting.

One of the most practical labels for RIA is ^{125}I. Its half-life of 60 days is sufficiently long to make its use feasible and permits a specific activity 100

times as great as with tritium. There is the further advantage that it is a gamma emitter which can be counted in the scintillation well counters available in most institutions. The radioiodine labeling generally can be accomplished directly in the case of all protein, hormone, and enzyme ligands. In the case of digoxin the tritiated compound was used initially and necessitated liquid scintillation counting. This involved inherent significant expense for sample preparation (special counting vials and solvent) and problems incidental to variation in quenching and chemiluminescence from sample to sample. In July of 1971 the Schwarz/Mann Company introduced a ^{125}I-labeled derivative of digoxin. In this instance an iodo-tyrosine moiety was chemically attached by means of an N-succinyl esterification link to the hydroxyl group on the Number 3 carbon of digoxigenin. This derivative despite its somewhat gross difference from digoxin, reacts almost indistinguishably from digoxin with the antidigoxin antibody. Its use in the digoxin RIA appreciably facilitates counting sample preparation, reduces expense, and should permit higher levels of radioactivity with consequent saving of counting time.

Solutions of labeled ligands for use in RIA present the same adsorption problems as were mentioned earlier with relation to the solutions of antibody. The solutions are necessarily so dilute with respect to the ligand that the ligand must be protected from adsorption onto the walls of containers. This may be accomplished by the use of coated glassware, plastic containers, and the addition of protective proteins to the solutions. Beyond the usual problems of stability of chemical solutions, high specific activity radioactive ligands are also susceptible to radiochemical breakdown. This can result in detachment of the radioactive label from the original ligand molecule with the result that the radioactivity does not follow the expected binding reaction and results of the test can become invalid. In the normal performance of RIA such problems are quite effectively monitored by the blank and digoxin standard tubes in each analytical run.

Standard ligand. The third requirement for RIA is a supply of standard or calibrated ligand for use in calibrating the assay procedure. In the case of digoxin, very high grade, pure crystalline compound is available and standard concentrations can be prepared by dilution from weighed material. Even this presents some problems in the case of digoxin because of its insolubility in water. It is usually necessary to prepare an initial concentrated solution in 30-50% ethanol with further subdilutions being made with water. The liabilities of working with extremely low-concentration solutions must again be emphasized, as it is possible to lose a significant fraction of the digoxin content through adsorption on container walls when the concentration is a few nanograms per milliliter. In our own experience we have compared the Schwarz/Mann standard digoxin with similar preparations made by Burroughs-Wellcome and with our own preparation from highly purified crystalline digoxin and have found consistently good agreement. In the case of the other RIA procedures, particularly in the case of hormones, it is often difficult to obtain reliable standardized material and in many instances the calibration of material must be based on bioassays.

Separation technique. A final requirement for RIA is the existence of an effective and simple separation technique to divide the reaction products into the free and bound fractions. These techniques must be highly efficient and result in essentially 100% complete separation. They must be sufficiently simple to be readily applicable to large number samples. The variety of applicable processes includes the use of resins, absorbants (charcoal), precipitation or binding by use of a second antibody, electrophoresis, paper and TLC chromatography, dialysis, ultrafiltration, and molecular sieving (Sephadex).

In the case of Schwarz/Mann digoxin immunoassay an adsorption technique using charcoal is employed. This, however, is not simple charcoal adsorption, but in effect further uses what amounts to a molecular sieve (3). The exterior surfaces of individual charcoal granules are coated with particles of large molecular weight (80,000) dextran. This results in a porous charcoal adsorbant, the surface of which is covered by adsorbed dextran. No charcoal binding sites can be reached through the dextran coat by the large digoxin-antibody complexes. There are small holes, however, between the dextran molecules, and these are sufficiently large to permit the small free digoxin molecules to penetrate the dextran coat and find binding sites on the walls of the small channels within the charcoal particles. In practice, the charcoal particles are coated with the dextran by simple exposure to a solution of dextran in which they are stored as a concentrated suspension which is diluted with buffer immediately before use. Because the working solution is in reality a suspension of dextran-coated charcoal (DCC) particles, it must be kept on a stirrer to maintain a uniform suspension through the time that it is being used in a given RIA run.

Radioimmunoassay Kits

In recent years RIA has become a commercial business and kits with all the necessary reagents are

Table 4. Schwarz/Mann Digoxin Assay

1.	Add	1.0	ml	buffer
2.	Add	0.05	ml	serum
3.	Mix			
4.	Add	10	μl	labeled digoxin
5.	Mix			
6.	Add	10	μl	antiserum
7.	Mix			
8.	Stand	30	min	
9.	Add	0.5	ml	DCC suspension
10.	Mix			
11.	Stand	10	min	
12.	Centrifuge	10	min	
13.	Decant into counting tube			
14.	Count			

currently available for over a dozen different RIA procedures. At present both Schwarz/Mann and Burroughs-Wellcome are manufacturing kits for the RIA of digoxin. In our own institution we have had over 2 years of experience with the Schwarz/Mann reagents and have used over 50 kits.

The step-by-step procedure for the Schwarz/Mann digoxin is given in Table 4. The reactions are carried out in 12 x 75-mm polystyrene tubes to avoid the surface adsorption effects that might be experienced in glass test tubes. Each sample is run in duplicate. The Schwarz/Mann test is conducted on a semi-micro scale in that reagents are used in 10-μl quantities. This is made practicable through the use of several sophisticated pipetting devices. The 50-μl samples of patient serum are measured using a Biopette (Beckton, Dickinson, and Co.) which delivers the required quantity by push button and uses disposable plastic tips. The several mixing steps are accomplished by the use of a mechanical rotary vortex mixer. The repetitive delivery of 10-μl quantities of labeled digoxin and antiserum are conveniently accomplished by using Hamilton push-button repeating microliter syringes (PB600, Hamilton Co.). This syringe in the 500-μl size can reliably deliver fifty 10-μl aliquots of reagent from its long hypodermic needle tip to the inner walls of the plastic reaction tubes.

The standing times at Steps 8 and 11 (Table 4) of the procedure do not seem to be critical and do not require a rigorous schedule for a series of up to at least 50 tubes. The addition of the DCC suspension at Step 9 has been accomplished using the Biopette making individual 0.5-ml deliveries from a beaker standing on a magnetic mixer. The centrifuging at Step 12 does not require 5,000 rpm or a refrigerated centrifuge as recommended by Schwarz/Mann but can be done in a 24-place head, table-model clinical centrifuge using 10 min at a full speed of approximately 2,500 rpm.

In our experience the most difficult step in the Schwarz/Mann digoxin RIA is the quantitative decanting of the supernate from the reaction tube into the gamma counting tube. This involves the successful transfer of approximately 1½ ml of aqueous solution from the small plastic reaction tube to a 14 x 150-mm plastic gamma well counting tube without loss, holdback, or spill-over of charcoal. The problem arises from the propensity of the aqueous solution not to wet the plastic; the last drop has a great tendency to remain behind. With experience a technician learns to accomplish this transfer by direct pouring followed by gentle tapping of the small tube lip against the inner rim of the larger tube to effect transfer of the last drop.

In our procedure the minimum test run, to assay a single patient serum, involves the use of eight tubes. Two tubes are used as blanks and contain no antiserum. These provide the effective background count which includes environmental background and a small contribution of ^{125}I which carries through into the supernate despite the charcoal removal step. On 28 successive Schwarz/Mann kits our blank counting rate proved to be 190 ± 30 cpm. The value is usually quite constant within one kit. A second two tubes represent zero digoxin level and contain normal human serum free of digoxin (Monitrol I·X, Dade Division American Hospital Supply Corp.). These tubes show the maximum counting rate and establish the zero digoxin end of our calibration curve. A third set of two tubes is prepared with Monitrol serum plus 5-ng equivalent standards of digoxin. These are used to provide the upper end of the calibrated region of the curve and would actually represent toxic serum levels of digoxin in any adult. The last two tubes are duplicate samples of the patient's serum. This minimum test run including one patient, and the necessary minimum controls requires about 2 hr for total analysis and counting. When the series is expanded to run 15 patient sera against the same controls, working time is approximately 3 hr. It has been our practice to use Monitrol as our normal human serum standard because it is reliably free of digoxin and radioactivity which could always be a concern in local blood bank pool serum.

Each time a new kit is received and placed into use, a full calibration curve is run using 0.4, 1.0, 2.0, 3.0, 5.0, and 10 ng digoxin standards in duplicate. Once these values have been confirmed to yield expected values, in conformity with past kits, the kit is accepted and subsequent daily determinations use only a 0.4- and 5-ng standard to bracket the normal range. Because of our Olivetti 101 calculator program, the 0.4-ng standard is calculated as an unknown and accepted if the determined value lies between 0.3 and 0.5. Figure 3 shows the calibration data for a digoxin run plotted in two different formats. The upper curved line represents

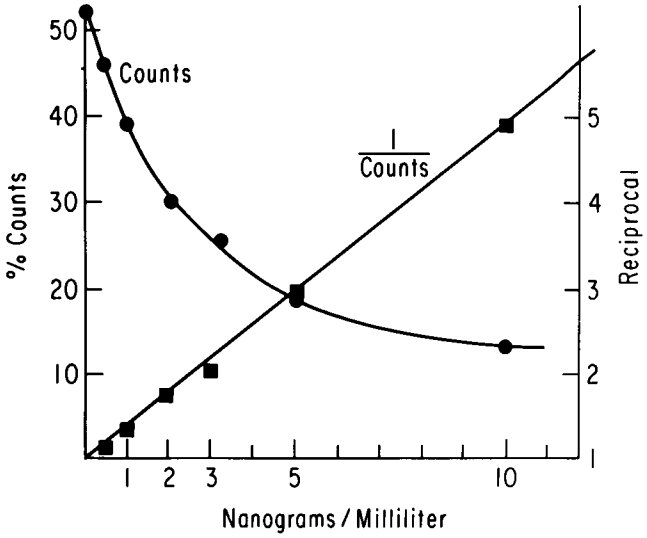

FIG. 3. Digoxin radioimmunoassay calibration curves. Curvilinear plot represents counting activity corresponding to various levels of digoxin standard expressed as percent of total ^{125}I activity in labeled digoxin derivative used per tube in analysis. Rectilinear relationship arises from plotting reciprocals of activity corresponding to each digoxin level expressed as decimal fraction of activity in zero digoxin tube.

the percent of the total counts in a digoxin test tube that was found as a function of the amount of digoxin standard present in the analytical sample. Normalizing the counts to a percent of the total counts present in the system permits direct comparison of values despite radioactive decay and variation from kit to kit. Such a curvilinear relationship can be used directly to translate the activity in an unknown test sample to nanograms per milliliter concentration of digoxin in the serum involved.

In absolute counts our 2-in. NaI (T1) scintillation well counter normally affords 3,000 cpm on the sample containing zero digoxin and approximately 250 net counts per minute on samples containing digoxin equivalent to 10 ng/ml. The straight line plot on Fig. 3 is the relationship between the reciprocal of the percent of total counts and the concentration of digoxin. In our routine calculations the counting rate of the zero digoxin standard is used as the denominator to which all other counts are normalized as percent and subsequently converted to the reciprocal of this decimal fraction. This approach yields values for the slopes of the line which should be consistent from day to day and independent of radioactive decay and the absolute level of activity in a kit. This linear relationship definitely appears to be somewhat in error for digoxin levels below 1 ng/ml with the error amounting to about a 10% underestimate of the level at 1 ng and as much as 20% at 0.5 ng. Since these deviations from the true value seem small in terms of their clinical significance, we have elected to use this simple regression line type of calculation in preference to reading values from a truly correct curve or going to a more elaborate computer calculation.

For day-to-day computation of results a program has been written for the Olivetti Underwood Programma 101 calculator by Nicholas Kutka of our group. The approximately 100 steps to this program are shown in Figure 4. In addition to calculating the digoxin levels for each patient, this program provides a percent standard deviation for the duplicates involved in each test to permit easy recognition of samples that do not show acceptable agreement. Figure 5 reproduces an annotated example of the program printout. Sample tubes 5 and 6 which this program calculated to have 0.89 ng/ml was in reality a 1.0-ng standard.

Serum digoxin RIA has been on-line as a routine test in our institution for 20 months. From essentially every aspect our experience has been favorable. The test was initially offered on a twice a week basis with results available the same day. Under these conditions approximately 100 tests per month were done for the first 3 months. The frequency of test performance was then increased to 3 times a week and demands for the test seem to be reasonably constant at about 150 tests per month in this 800-bed general hospital. Due to the cost associated with running controls and standards, it appears excessively expensive to do less than 5-10 chargeable tests per run. This has governed the frequency with which tests are performed in our institution and together with personnel problems has led us not to undertake emergency determinations. The out-of-pocket costs of supplies is approximately $1.00 per tube. Since eight tubes are required for a minimum one patient determination this involves approximately $8.00 of supply expenses. There is another approximately $20.00 cost associated with the performance of a full calibration curve for a new kit. The Schwarz/

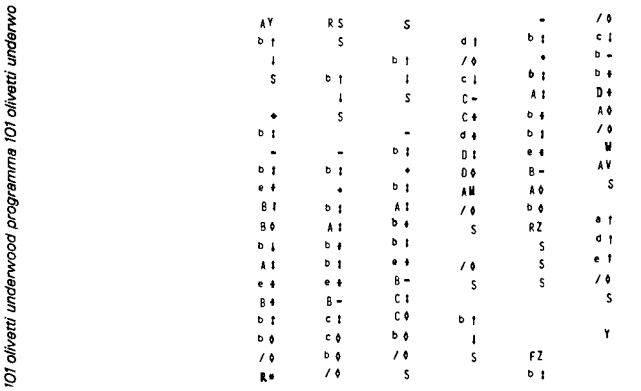

FIG. 4. Tabulation of program steps for producing magnetic program card for digoxin radioimmunoassay calculations on Olivetti Programma 101 electronic desk computer.

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	Operator Entries*	Olivetti Print	
Start program by depressing "V"			V
Enter date, e.g. 7. 21 1972	*Date	07•211972	S
Enter blank tube counts (2)	*Counts	678	S
	*Counts	691	S
Prints average of blank counts		684•500	B◊
Prints standard deviation of duplicates (691 - 678) ÷ 684.5 ÷ 2 %		0•009	b◊
Enter zero digoxin counts (2)	*Counts	7918	S
	*Counts	7635	S
Prints average net counts		7092•000	c◊
Prints standard deviation %		0•018	b◊
Enter 5 ng (or other) digoxin standard Counts (2)	*Counts	3123	S
	*Counts	3072	S
Prints average net counts		2413•000	C◊
Prints standard deviation %		0•008	b◊
Enter 5 ng (or other) in standard	*Nanograms	5	S
Prints SLOPE		0•387	D◊
PROGRAM IS READY----------			
For each patient sample: Enter tube numbers (2) e.g. # 5 & 6	*Tube Nos.	5•6	S
Enter patient counts (2)	*Counts	5938	S
	*Counts	5976	S
Prints average net counts		5272•500	A◊
Prints standard deviation %		0•003	b◊
1st patient result in ng/ml	RESULT	0•891	A◊
2nd patient, tube numbers (2)	*Tube Nos.	09•10	S
2nd patient, counts (2)	*Counts	4355	S
	*Counts	4511	S
Prints average net counts		3746•500	A◊
Prints standard deviation %		0•017	b◊
2nd patient result in ng/ml. etc.	RESULT	2•302	A◊

FIG. 5. Annotated sample digoxin radioimmunoassay calculations as produced by program given in Fig. 4 on Olivetti Programma 101. Program is initiated by depressing "V" key after program has been entered into computer by magnetic program card. Each count entry called for is entered by operator as gross observed count for any uniform counting time for all tubes, e.g. 1, 3, 5, or 10 min per tube.

Mann kit nominally provides 240 tubes which at our level of operations suffices for 50 ± 10 patient tests per kit. It would appear necessary to have close to 100 tests per month for this test to pay its way.

Clinical Considerations

The serum digoxin RIA has produced results which have been favorably received by clinicians and which have caused minimal problems for those concerned with the conduct of the test. We began with the assumption that the normal values given by Smith and Haber (Fig. 6), would be applicable and expected that our normal values should fall in the 1.4 ± 0.7-ng/ml range (*4*). For the first 4 months the test was in use, a physician from the Division of Nuclear Medicine personally investigated all abnormally high or low values to determine whether there were plausible reasons for the abnormal values. This proved to be a very satisfactory approach to both quality control and to promoting acceptance of the test by the clinician us-

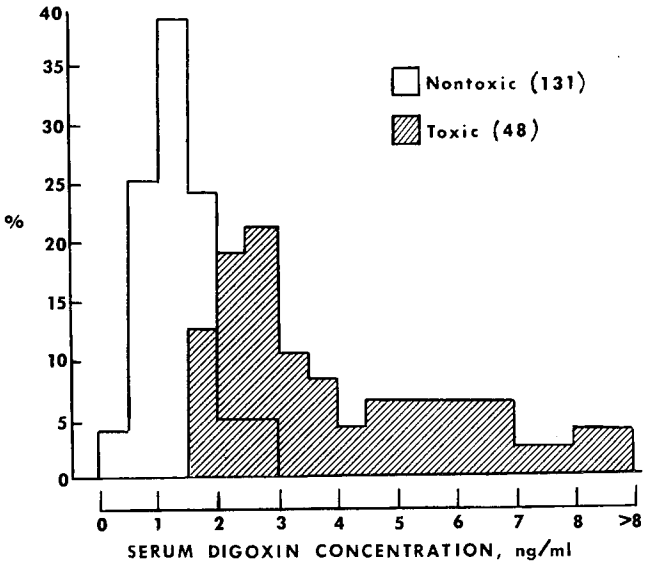

FIG. 6. Frequency histogram showing proportions of nontoxic and toxic patients with serum digoxin concentrations in ranges shown. Ninety percent of patients with no evidence of digoxin intoxication had levels of 2.0 ng/ml or below, while 87% of toxic group had concentrations about 2.0. (Reprinted from J Clin Invest 49: 238, 1970.)

ers. In the vast majority of instances it was possible to determine that the serum level reported was in agreement with the circumstances of the patient. It is my personal conviction that such detective work by the physician responsible for the test is essential to promoting confidence on the part of the using physicians. For example, a clearly toxic blood level was found in one patient to be attributable to the extra "heart pills" given to the patient by his wife in a last effort to avoid having to bring him to the hospital, a fact of history that was not elicited by the staff physicians managing the case. Conversely we have had low-to-undetectable levels of digoxin in patients believed to be on daily doses only to find in the nurses notes that the dose was withheld for several successive days because of bradycardia found by the nurse at the time for the digoxin administration. In other instances inordinately high blood levels have been associated with patients having renal failure or with blood samples drawn too soon after the administration of a digoxin dose. Approximately 10% of the samples submitted for analysis by our clinical services fall in the toxic category, using 2.5-3.0 ng as the lower limits of probable toxicity as a criterion. In almost all of these cases a careful clinical assessment or the subsequent course of events has tended to confirm that the patient was toxic. Conversely, low or undetectable levels of digoxin have been found in patients in whom it was ultimately possible to establish that they had probably not been taking the drug, were on digitoxin instead of digoxin, or had apparently bona fide malabsorption.

Fogelman has challenged the utility of serum digoxin levels (*5*), but his analytical methods were not the same as we are using and a subsequent article by Doherty has perhaps appropriately pointed out that commercial supplies of faulty digoxin tablets may have been responsible for some of Fogelman's findings (*6*). We have had no indication in our experience of other drugs causing cross-reaction in the serum digoxin RIA.

We have been disturbed by a few cases in which our computer program calculated a negative serum digoxin level. This simply means that the patient test samples showed a higher counting rate than our Monitrol blank and probably indicates that some drug or metabolite has interfered with the charcoal adsorption step of the test. This finding has been observed by others and in our own laboratory recovery experiments using known quantities of digoxin added to such sera have shown unequivocal interference with the assay. We have not as yet been able to correlate this with any specific medication or metabolic derangement. We have had one instance of interference with a test by radioactivity administered to the patient for another purpose, specifically 100 mCi of ^{131}I for treatment of thyroid carcinoma 5 days before the digoxin blood sample.

With respect to the Schwarz/Mann digoxin RIA kit performance our experience has been favorable. We would prefer that the activity level in the ^{125}I digoxin derivative be somewhat higher than it has been to date. With freshly delivered kits our counting rate on the zero digoxin samples has been approximately 2,400 cpm. The 5-ng standards show approximately one third of this and for this reason it has been necessary to count all samples for a minimum of 3 min to achieve a 2% counting error with 0.95 probability. The slope of the reciprocal of the percent of counts per minute has varied from 0.31 to 0.48 slope units per nanogram in 30 kits evaluated. On repeat determinations with materials from the same kit the slope has been reproducible within about 5% of its value. We have come to expect duplicate analytical tubes to yield values within 5% of one another and at 10% disagreement we reject the test. An ^{129}I standard is used as a day-to-day instrument and counting efficiency reference.

The digoxin radioimmunoassay appears to be a useful, reliable, and thoroughly practical test which is economically feasible as a service to be provided on a routine basis by the nuclear medicine service in any general hospital facility having 200-300 or more beds.

Summary

A general background is given of the factors involved in radioimmunoassay with a detailed discussion of the specific application to serum digoxin analysis as a model. The practical aspects of the technique are emphasized. The favorable experience with providing digoxin assay as a routine clinical test using commercially available materials is described.

References

1. Smith TW, Butler VP, Haber E: Determination of therapeutic and toxic digoxin concentrations by radioimmunoassay. *New Engl J Med* 281: 1212-1216, 1969

2. Smith TW, Butler VP, Haber E: Characterization of antibodies of high affinity and specificity for the digitalis glycoside digoxin, *Biochemistry* 9: 331-337, 1970

3. Herbert V, Lau K, Gottlieb CW,et al: Coated charcoal immunoassay of insulin. *J Clin Endo* 25: 1375-1384, 1965

4. Smith TW, Haber E: The clinical value of serum digitalis glycoside concentrations in evaluation of drug toxicity. In *Drug Metabolism in Man, Vesel ES, ed, Ann NY Acad Sci* 179: 322-337, 1971

5. Fogelman AM, Finkelstein S. LaMont JT, et al: Fallibility of plasma-digoxin in differentiating toxic from nontoxic patients. *Lancet* 2: 727, 1971

6. Doherty JE: Letter to Editor, Digoxin availability "like it is". *New Engl J Med* 286:266, 1972