Radioimmunoassay for Digoxin with a Fully Automated Continuous-Flow System

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We describe a fully automated continuous-flow radioimmunoassay for digoxin with use of a Technicon AutoAnalyzer system. It makes use of antibodies linked to magnetizable particles; a magnet separates the bound and free fractions. The precise dispensing of reagents by the AutoAnalyzer system permits a small incubation volume (about 160 μl) and the reproducible timing enables non-equilibrium conditions to be used. Thus a single sample is assayed in 15 min, with a sample throughput of 30/h. The standard curve ranges from 0.5 to 8.0 μg/liter and is most precise between 1 and 3 μg/liter, the between-assay coefficient of variation being less than 3%. There is no significant carryover between samples of high and low concentration, and results by the method correlate closely (r = 0.969) with those by an established manual assay in which charcoal separation is used.

Previous attempts to develop automated radioimmunoassay systems based on continuous-flow principles have been largely unsuccessful, due in part to problems encountered in separating the antibody-bound and free fractions and to marked carryover associated with the use of long delay coils used in an attempt to enable the reaction to approach equilibrium.

An assay for insulin described by Pollard and Waldron (1) involved a 210-min incubation, followed by filtration of the second-antibody-precipitated bound fraction on a continuous strip of reinforced glass-fiber paper. Bagshawe et al. (2) used a similar approach, with a 120 min incubation, for assay of human choriongonadotropin and lutropin. Johnson et al. (3) and Luner (4) also developed assays for insulin in which the elaborate mechanical separation units of the earlier systems were replaced by a sedimentation technique. Thus, Johnson et al. decanted the bound fraction after second-antibody precipitation, whereas Luner used antibodies attached to erythrocytes which, after incubation, were aggregated with “Polybreen” and separated by sedimentation.

More recently a continuous-flow unit has been developed (Auto Assay Inc.) that incorporates a packed column of antiseraum attached to solid phase. Designed mainly for the assay of extracted steroids, the system has a low throughput rate, with an incubation time of about 15 min. In another recently described continuous-flow system, the “Gammaflow” (5), an ion-exchange resin column is used to separate the bound and free fractions. It has a 5 min throughput time and an analysis rate of 20 samples per hour for digoxin, with a correlation coefficient of r = 0.75 when compared with a manual digoxin assay.

The system we describe is constructed from modified AutoAnalyzer units and makes use of antibodies covalently linked to microfine magnetizable particles. Bound and free fractions are completely separated by applying an external electromagnetic field. Nonequilibrium conditions (with only 10-min incubation per sample) enable short incubation coils to be used with the consequent elimination of carryover between samples. The experimental system runs at 30 samples per hour for digoxin, and precision and accuracy have been extensively studied. The results obtained for patients’ samples have also been compared with those of an established manual, liquid-phase assay.

Materials

Digoxin antiserum: High-titer antiserum was raised in rabbits against a digoxin/bovine serum albumin conjugate prepared according to the method of Smith et al. (6).

Labeled digoxin: 125I-labeled tyrosine-methyl ester of digoxin (spec. act. 150 Ci/g) was obtained from Burroughs Wellcome, Beckenham, Kent, U. K. This was diluted in phosphate buffer (0.05 mol/liter, pH 7.4) to give a concentration of 50 pg/μl for the automated assay and 100 pg/50 μl for the manual assay.

Digoxin standards: Crystalline digoxin (European Pharmacopeia) was dissolved in ethanol, diluted in phosphate buffer (50 mmol/liter, pH 7.4) and further diluted in digoxin-free plasma to give concentrations of 0.5, 1.0, 2.0, 3.0, 4.0, and 8.0 ng/ml (μg/liter). These were divided and stored at −20 °C until required.
Fig. 1. System flow diagram

Methods

Manual Assay

We used a modification of the assay previously described by Greenwood et al. (7). To a 200-μl plasma sample or standard, in duplicate, was added 50 μl of 125I-labeled digoxin (100 pg), followed by 50 μl of a 4200-fold dilution of rabbit antiserum (resulting in about 60% binding of labeled digoxin in the absence of cold). After 45 min, free and bound fractions were separated by adding a dextran-coated charcoal suspension (5 mg/400 μl) followed by centrifugation. Radioactivity in the free fraction, adsorbed to the charcoal pellet, was counted and the percentage bound counts was calculated and plotted vs. the digoxin concentration in the standards.

Automated Assay

Preparation of the magnetizable particles: Finely divided iron oxide was incorporated into a polymeric matrix by standard techniques. After they were ground to a suitable size, the particles were sedimented with a magnet and the supernate was aspirated. They were then washed with 5–6 liters of cold distilled water until the pH was decreased to about 7.2 and finally with buffer before being coupled to the γ-globulin fractions from rabbit antiserum or normal serum, prepared by precipitation with anhydrous sodium sulfate (0.18 g/ml). Coupling was achieved by suspending the activated magnetic particles in 0.1 mol/liter bicarbonate buffer (pH 8.6) and adding the partly purified globulin fractions, followed by vertical rotation for three days at 4 °C. The solid phase was then sedimented on the magnet and the supernate aspirated. Finally, the particles were washed at room temperature with buffer, then stored at a concentration of 1 g/10 ml in phosphate buffer at 4 °C. Under these storage conditions the binding capacity did not significantly decrease during several months.

Description of the AutoAnalyzer System

A flow diagram of the hydraulic system is shown in Figure 1 and has been described fully elsewhere (8). It includes a Technicon AutoAnalyzer Sampler IV, electronically controlled by a timing programmer to give the required sample-to-wash ratio (1/2) and cycle frequency (30/h); a proportioning pump; glass mixing and time-delay coils; and transmission tubing and Tygon and polyethylene tubing.

Solenoid air valves, operated by the programmer, and linked to pneumatic pinch valves, are used to discretely introduce label and antibody to the sample, and to control the flow of particles to the counter.

A timing sequence programmer is used to activate the sampler and control the phased addition of label and antibody to the sample. This “sample cycle” is initiated by the operator and is automatically repeated throughout the run. The programmer also controls a “separation cycle.”

Introduction of label: Label, contained in a lead shielded reservoir, is pumped via a pinch valve (valve 1, Figure 1) either to the analytical system or circulated back to the reservoir. The valve is operated by switching air pressure to the top chamber of the valve, which allows label to be added to the sample segment as a discrete aliquot. When label is recycling, a compensating buffer flow controlled by the same valve is entering the analytical system, thus ensuring a constant flow. The time and duration of label addition to the system is controlled by the programmer so that on entering the
analytical system the label is phased to meet and mix with the sample in the micro mixing coil, at point A in Figure 1.

Introduction of solid phase antibody: A discrete addition and recycling system, similar to that used for label, is used for the timed addition of antibody to the premixed sample and label segment (point B, Figure 1). The solid phase is kept in suspension by constant stirring and the particle stream is air segmented on leaving the reservoir to prevent settling in the recirculation loop.

Phasing: Pre-determined delays and addition times for label and antibody are programmed into the timing unit to enable these reagents to be accurately phased to the sample segment. Since antibody and label must not mix in the absence of serum, a pyramid type phasing pattern is adopted which enables an injection of label (55 μl) to be added to the center of the sample segment (60 μl). After mixing, antibody (50 μl) is added to the center of the combined sample/label segment.

Incubation: The air-segmented samples with added label and the magnetizable particles are incubated at ambient temperature in a 20-turn glass mixing coil that gives a 10-min incubation period. Each sample is separated from the next by intersample wash buffer aspirated by the sample probe.

Separation of the bound and free fractions: On leaving the incubation coil, the magnetic particles flowing through a light-activated detector switch activate a pair of electromagnets positioned in sequence. The particles are retained by the first magnet and washed by the ensuing buffer stream and extra added wash buffer (1 ml) for 1 min, after which time the first magnet is switched off and the particles are passed to the second magnet, where they are held and washed for a further 15 s. When the second magnet is de-energized the particles are transferred via a pneumatic pinch valve (timed to open when the second magnet is switched off) to a double spiral 80-s delay coil in the counter well. The on-line counting and printout procedures are automatically controlled by the counter, the electronics unit from a commercially available gamma counter linked to a teletype being used.

Development of the Assay Procedure

Sample: A total incubation volume of about 160 μl with a sample volume of about 60 μl was selected.

125I-labeled digoxin: Label concentration was varied until the lowest count recorded over the range of the standard curve was about 8000 to 10 000 counts per 80 s (for the 8 ng/ml standard), thereby minimizing the statistical counting error. A 50 pg/μl concentration of label was finally selected and was compatible with the sensitivity required of the standard curve.

Antibody-linked magnetizable particles: A concentration of magnetizable particles was chosen to give a standard curve with optimum precision in the clinically important range. Standard curves were run with various dilutions of the magnetizable particles and a 1/26 dilution of the stock was finally chosen. This very low concentration of magnetizable particles (0.18 mg/50 μl) was inadequate to trigger the light-sensitive switch reliably and the antibody-bound particles were subsequently diluted in normal rabbit IgG-linked particles (1.5 mg/50 μl) to increase particle density.

Incubation time: Figure 2 shows the progress of the reaction for the zero and top standards (8 ng/ml) as a function of incubation times ranging from 34 s to 24 min. Steady state is reached after 17 min, and after 10 min the reaction has attained 85% of the equilibrium value. Precision is comparable throughout the range of the curve, with CV's of 1.9–2.8% (n = 6) measured at each point. For convenience and compatibility with other assays run on the system (thyroxine, triiodothyronine, triiodothyronine uptake, human somatomammotropin, and cortisol) a 10-min incubation period was selected, though much shorter times could be used. Figure 3 shows standard curves obtained for 4-, 10- and 24-min incubations, the counts being plotted as a percentage of the maximum counts obtained in the absence of unlabeled digoxin (C0) vs. digoxin concentration in the standards. There was no significant difference in the standard curves, with excellent precision in each case.
Table 1. Automated Assay Precision for Four Samples

<table>
<thead>
<tr>
<th>Within-assay</th>
<th>Between-assay</th>
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<tr>
<td>Mean (ng/ml)</td>
<td>Mean (ng/ml)</td>
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<tr>
<td>SD</td>
<td>SD</td>
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<tr>
<td>CV, %</td>
<td>CV, %</td>
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<tr>
<td>0.83</td>
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<td>0.04</td>
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<td>2.9</td>
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Use of different antisera: Standard curves were obtained with antisera raised in a rabbit and a sheep. The results were comparable, with slightly greater sensitivity for the rabbit antisera.

Assay Procedure

A series of standards was aspirated, once at the beginning and once at the end of each assay, and a digoxin-free plasma pool was always sampled in duplicate at the start of each run, to give the maximum binding of 125I-labeled digoxin to the magnetizable particles (C0). The counts obtained were expressed as a percentage of the mean C0 and plotted vs. digoxin concentration in the standards. Drift was monitored by inserting quality-control standards once every five samples.

Results

Precision and accuracy: The “within” and “between” assay CV’s, determined by repeated assay of plasma pools with digoxin concentrations covering the clinically imported range, were determined for both the automated and manual assays.

The within-assay values for the manual assay lay between 2.4 and 5.4% (n = 10); the between-assay results lay between 2.7 and 7.3% (n = 10). Somewhat better precision (Table 1) was attained with the automated system, which gave good within-assay values of 2.9-4.5% (n = 10) and between-assay results of 5.4-5.6% (n = 10).

The precision of both assays was reflected in analytical recovery experiments in which we used three different (digoxin-free) sera, with digoxin added at two concentrations (1 and 3 ng/ml). The mean recoveries lay between 98-105% for the automated assay, 93-99% for the manual assay.

Correlation: Results for 50 samples from patients receiving digoxin, assayed by the automated method and by the liquid-phase manual assay, showed a linear relationship (Figure 4), with a correlation coefficient of 0.969.

Specificity: The specificity of the antibody in the liquid phase and after coupling to cellulose magnetized particles was tested by assaying solutions of testosterone, estradiol, cortisol, progesterone, cholesterol, and spironolactone, prepared in charcoal-treated serum, over the concentration range 10 to 10,000 nmol/liter. The results for the automated assay (Figure 5) demonstrate that there was no cross-reaction with the natural steroids at concentrations in excess of those encountered physiologically. Only spironolactone showed any measurable cross-reaction, and this at concentrations far in excess of those found in patients taking the drug.

Protein effect: The antibody binding of 125I-labeled digoxin was studied in 30 digoxin-free sera with protein concentrations in the range of 50-85 g/liter (albumin 10-50 g/liter). The mean percentage binding of the labeled digoxin had a CV of 2.1% with the automated system, 2.27% with the manual system. Thus there was no significant effect on either assay attributable to varying protein or albumin concentrations.

Automated Assay

Carryover: The system was tested for carryover from a sample of high to one of low digoxin concentration by repeatedly analyzing 8.0 and 0.5 ng/ml standards, alternately and in duplicate. This was done on two occasions and no carryover was observed, the CV being 3.0% at 8.0 ng/ml on both occasions and 1.0% and 2.0% at 0.5 ng/ml.

Assay drift: We studied the drift in the AutoAnalyzer system during the course of an assay run by inserting an aliquot of pooled digoxin plasma after every fifth
sample. The mean CV for the results was 1.5%. Standard curves run at the beginning of an assay and again 270 min later, at the end of a run (after 130 samples) were superimposable. Thus assay drift was not a problem in this system. The high reproducibility of the standard curve is illustrated in Figure 6, which shows the range of values for 10 standard curves run on 10 consecutive days.

Manual Assay

Separation: The time dependence of the charcoal separation procedure was investigated by incubating labeled digoxin and antiserum in digoxin-free plasma with charcoal (5 mg/400 μl) for increasing periods of time before centrifugation. The binding was constant for 10 min (64% at 10 min) and then began to decline slowly (60% at 40 min and 57% at time 120 min). Thus assays were kept to such a size that separations could be done within 10 min.

Discussion

A highly precise assay for digoxin has been developed using the prototype of a fully automated continuous-flow system. The long-term stability would enable emergency digoxin assays to be performed without necessarily always running a full standard curve, which is of particular importance for digoxin, where rapid results can be vital in cases of digoxin toxicity. For convenience, a 10-min incubation time (15 min throughput) and a rate of 30 samples per hour is used, although shorter incubation times and faster rates are possible.

Carryover, which was a major drawback in the earlier continuous-flow systems, resulted from the long incubation coils we used in an attempt to attain equilibrium. It has been eliminated in the present system by using nonequilibrium conditions and short incubation coils. This nonequilibrium approach is made possible by the highly precise timing of reagent addition and separation that the AutoAnalyzer offers. Thus high precision is retained over the range of the standard curve, even with an incubation period as short as 5 min.

The on-line washing procedure ensures complete separation of the bound and free fractions and, as expected, assay precision is considerably improved as compared with the manual system. The correlation obtained with both systems is excellent.

Error reported for digoxin radioimmunoassays is mainly due to the use of labeled preparations of poor antibody affinity and of antibodies of poor specificity and avidity, which can lead to varying results, depending on serum protein concentration (9) and the nature of the separation procedure, particularly when the time-dependent charcoal separation method is used. These difficulties have been minimized by using the 125I-labeled tyrosine-methyl-ester of digoxin (rather than the 3-O-succinyl-digoxigenin 125I-tyrosine preparation), which has an immunoreactivity comparable to that of digoxin itself. In addition, the rabbit antiserum chosen for use in these assays is highly specific for digoxin and its cardioactive metabolites, shows high avidity binding, as demonstrated by the time independence of the charcoal separation procedure, and is not sensitive to variations in sample protein concentration, either in the liquid or solid phase systems.

References