Fluoroimmunoassay of Digitoxin in Serum
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This fluoroimmunoassay for digitoxin in serum involves use of a sheep antiseraum to digitoxin coupled to magnetizable solid-phase particles and fluorescein-labeled 3-O-succinyl digitoxigenin as tracer. Sodium salicylate blocks binding of the drug by binding proteins, and endogenous fluorophores and other interfering components in serum samples are reliably and completely removed at the separation and wash steps, which are facilitated by magnetic sedimentation. The method is sufficiently sensitive, precise, and specific for application to routine monitoring of digitoxin therapy, and results correlate closely (r = 0.992) with those of an established radioimmunoassay.

Additional Keyphrases: drug assay • magnetizable solid-phase particles • heart disease

Digitoxin is a cardiac glycoside used mainly in Germany and Scandinavia and, to a lesser extent, in North America and the rest of Europe for treating congestive heart failure and certain cardiac arrhythmias (1). Circulating concentrations of the drug correlate with therapeutic effectiveness and with toxicity, the toxic effects associated with the cardiac glycosides being among the most serious encountered in clinical practice (2-6). Significant variations in the absorption of orally administered cardiac glycosides (2) make it difficult to predict the dose required to attain circulating concentrations within the narrow therapeutic range (usually, 10-30 μg/L). Furthermore, their slow rate of metabolism and excretion may lead to toxic values. Correct dosage is a particular problem in patients with impaired renal function (2). Digitoxin concentrations in serum are most commonly determined by radioimmunoassay involving 3H or 125I as the label. Enzymoimmunoassays of the separation (7-9) and the nonseparation (9-12) type have been developed for digitoxin, but have not been widely applied to assay of digitoxin. Endpoint measurement of enzyme activity prolongs an immunoassay and involves extra reagent additions and (or) manipulations.

In the present fluoroimmunoassay (FIA) for digitoxin in serum, a fluorescein-labeled digitoxin derivative is used as tracer. The fluorescence can be measured simply, precisely, and rapidly, and the long shelf-life of the reagents is an advantage for an assay that may be required only infrequently.

Materials and Methods

Reagents

Sources: Fluorescein isothiocyanate isomer I (FITC), digitoxin, digitoxigenin, digoxin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, succinic anhydride, N-hydroxysuccinimide, and spironolactone were obtained from Sigma Chemicals Ltd., Poole, Dorset, U.K.; canrenone (17-hydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid lactone) and potassium canrenate from Searle, High Wycombe, Bucks., U.K.; sodium metaperiodate, disodium tetraphosphate, sodium salicylate, sodium azide, sodium hydroxide, methanol, and sodium hydrogen carbonate from British Drug House, Poole, Dorset, U.K.; bovine serum albumin (Fraction V) from Armour, Eastborne, Sussex, U.K.; silica gel thin-layer chromatography sheets (DC-Alufolien Kieselgel 50 P254) from Merck, Darmstadt, F.R.G.; and magnetizable cellulose/iron oxide particles from the Department of Chemical Pathology, St. Bartholomew's Hospital.

Buffers: Sodium borate (200 mmol/L, pH 9.5) containing 60 g of sodium salicylate, 1 g of bovine albumin, and 1 g of sodium azide per liter was the diluent buffer. We used sodium borate (20 mmol/L, pH 9.5) containing 1 g of bovine albumin and 1 g of sodium azide per liter for the wash steps.

Fluoroimmunoassay standards: We diluted with pooled normal human serum a 250 mg/L solution of digitoxin in ethanol and stored these standards at −20 °C.

Preparation of Digitoxin Tracer

Synthesis of 3-O-succinyl digitoxigenin: We used the method of Yamada (13). Dissolve 860 mg of succinic anhydride and 422 mg of digitoxigenin in 13.4 mL of pyridine. Protect from light and allow to react at room temperature for three months. Then pour the solution into 75 mL of cold H2SO4, 1 mol/L; filter; wash the solid product with cold water, and redissolve it in 150 mL of chloroform/methanol (2/1 by vol). Wash this solution once with 25 mL of H2SO4, 1 mol/L, and three times with 25 mL of water. Add 25 mL of methanol after each washing. Dry the organic phase over anhydrous sodium sulfate and evaporate it under a stream of nitrogen. Redissolve the residue in 15 mL of hot ethanol, then add hot water until the solution becomes turbid. After letting the solution cool at room temperature, keep it at 4 °C for 48 h. Filter, then wash the resulting crystals three times with cold ethanol/water (3/2 by vol). The final product is a white powder, mp 222-227 °C; yield is about 40%.

Coupling of 3-O-succinyl digitoxigenin to fluorescein-ethylenediamine: Dissolve 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (10 mg), N-hydroxysuccinimide (1 mg), and 3-O-succinyl digitoxigenin (5 mg) in 1 mL of N,N-dimethylformamide; stir the reaction mixture for 30 min. To the resulting active ester solution add, dropwise, 500 μL of a 5 g/L solution of fluorescein-ethylenediamine [prepared as described previously (14)] in dimethylformamide. After stirring for a further 60 min, apply the reaction mixture to silica-gel sheets (200 μL per sheet) and develop with chloroform/methanol (80/20 by vol) to separate a major product having an RF value of 0.64 (the RF value of fluorescein-ethylenediamine is 0.02). Scrape the area of the silica gel that contains the product from each sheet, elute each with 1 mL of methanol, and store the eluates in the dark at 4 °C.

To estimate the concentration of the product, dilute it in sodium bicarbonate buffer (50 mmol/L, pH 9.0) and measure the absorbance of the fluorescein group at 492 nm, at which wavelength its molar absorptivity is 8.78 × 104 L · mol−1 · cm−1 (14).

Digitoxin Immunogen

Prepare the immunogen by linking the digitoxin to bovine albumin by the periodate procedure (15). Dialyze the product against distilled water for three days, then lyophilize.
Anti-Digitoxin Sera

We immunized each of three sheep with 2.5 mg of immunogen in 4 mL of an emulsion of saline and complete Freund's adjuvant (1/3 by vol), apportioned among several intramuscular and subcutaneous sites. Six weeks later we re-immunized the sheep with 1.25 mg of immunogen in incomplete Freund's adjuvant; the animals were bled 10 days later. Further immunizations were made every two weeks and the animals were bled 10 days after alternate injections. We used antiserum from the third bleeding of one sheep in the studies described below. Suitable titers were obtained from all three sheep, however.

This whole sheep anti-digitoxin serum was coupled to magnetizable cellulose/iron oxide particles (1 mL/g) by the cyanogen bromide method as described previously (16).

Patients' Samples

Serum samples were obtained from 99 patients being treated with digitoxin. Concentrations of digitoxin were also determined with a commercial kit (Diagnostics Products Corp.) involving the use of a I25I-labeled tracer and polyethylene glycol/second antibody separation.

Apparatus

We used a multi-polar ferrite block magnet with a flat surface (approx. 150 x 200 mm; Magnet Applications, London, EC1, U.K.) and a microprocessor-controlled ratio-recording fluorometer (Fluororex; WilJ International, Ashford, Kent, U.K.) equipped with fluorescence filters (λex 490 nm, λem 520 nm) and a test-tube sampler adaptor.

Procedure

Assays were done at room temperature, in duplicate, in disposable polystyrene tubes (55 x 12 mm, no. 55.484; W. Sarstedt, Leicester, U.K.). To 500 μL of serum sample (or standard) add 100 μL of digitoxin tracer (30 nmol/L in diluent buffer), followed by 100 μL of anti-digitoxin solid-phase (6 g/L), and incubate for 60 min on a mechanical shaker. Then sediment the particles by placing the rack containing the assay tubes on the magnet for 2 to 5 min. Aspirate the supernatants to waste. Add 1.5 mL of wash buffer, vortex-mix the tubes' contents, then sediment the particles as before and aspirate the supernatants to waste. Finally, add 1.2 mL of eluton reagent (methanol/sodium bicarbonate buffer, 50 mmol/L, pH 9.0, in the proportion of 7/3 by vol), vortex-mix, re-sediment the particles on the magnet, and measure the fluorescence of the supernates containing the eluted bound fraction of the labeled digitoxin. In our laboratory the standard curve was automatically fitted and the results calculated and printed by the integral printer of the fluorometer.

Results

Optimization of Assay Conditions

Experiments performed in the absence of fluorescein-labeled digitoxin confirmed that endogenous fluorophores in serum were efficiently removed in the aspiration step of the assay. However, turbid or icteric serum samples required an additional wash at this step to ensure complete removal of endogenous interfering factors. Other than fluorescein-labeled digitoxin, the only significant contribution of the final signal was a background of <2% from the assay eluent. Therefore, we recorded total fluorescence signals; no blank or background corrections were necessary.

Sodium salicylate was required to block the binding of fluorescein-labeled digitoxin by serum proteins. In its absence, little of the tracer was bound by the antibodies in the presence of digitoxin-free human serum. Incorporating salicylate (60 g/L) in the diluent buffer restored antibody binding to the level observed in buffer alone. The ammonium salt of 8-anilino-1-naphthalene sulfonic acid (60 g/L) was also effective, but was not suitable for use because of its intrinsic fluorescence, which necessitated an additional wash of the solid phase before addition of the elution reagent.

Various proportions of methanol and sodium bicarbonate buffer were assessed for use in the elution of labeled digitoxin from the antibody-coupled solid phase. Efficiency was best (near 100%) when a proportion of 7/3 by volume was used.

At least 95% of the labeled digitoxin was bound by excess anti-digitoxin serum coupled to solid phase; nonspecific binding and entrapment of the tracer by the solid phase were less than 2%. On the basis of antiserum dilution curves, 0.6 mg of magnetizable particles per tube was sufficient to construct a standard curve that covered the clinically significant range of digitoxin concentrations. Figure 1 shows a typical standard curve.

Sensitivity: The minimal detectable concentration of digitoxin in serum at the 95% confidence level was 1.2 μg/L, and the minimal detectable amount (17) was 0.6 ng per tube.

Precision: To assess within-assay precision, we measured three serum specimens 10 times each, obtaining mean values of 5.2, 20.1, and 40.3 μg/L. The coefficients of variation (CVs) were 6.1, 3.2, and 4.1%, respectively. Assay of the same specimens on 10 different days gave between-assay CVs of 7.2, 6.1, and 5.3%, respectively.

Recovery: Known amounts of digitoxin were added to each of three patients' sera whose initial digitoxin concentrations had been determined by radioimmunoassay and FIA to be 10, 25, and 45 μg/L. Average analytical recoveries of 5, 20, and 40
\( \mu g \) of added drug per liter were 99, 101, and 102\% (n = 10).

**Specificity**: Cross reactivities of digoxin, lanatocide C, digitoxin, and ouabain in the digitoxin FIA were 60, 15, 1, and 0.02\%, respectively. No detectable cross reaction at a concentration in serum of 1 g/L was found with ami odorone, cholesterol, cortisol, disopyramide, estriol, spironolactone or its metabolites (canrenone and canrenenate), dexamethasone, valproic acid, phenytin, carbamazepine, or phenobarbital.

**Correlation with Radioimmunoassay**

Results of the assays of 99 patients’ specimens by FIA (y) and by an established radioimmunoassay (x, the Diagnostic Products kit) were related by the regression line \( y = 0.97x + 0.002 \), calculated assuming the two methods to have equal precision characteristics (18). The correlation coefficient \( (r) \) was 0.992, \( S_{yx} = 1.9 \mu g/L \).

**Discussion**

The separation step in an FIA, besides separating the antibody-bound from the free fraction before endpoint detection, can be used to remove endogenous fluorophores and other interfering factors in serum. This is particularly important when the concentration of an analyte is low (in the microgram per liter range), because the serum cannot be sufficiently prediluted to decrease the concentration of serum interferences to an insignificant amount.

Direct FIA of digitoxin in 500-\( \mu L \) serum samples is sufficiently reliable and sensitive for clinical purposes. Spironolactone (and, more importantly, its metabolites, canrenone and canrenenate) interferes in some immunoassays for cardiac glycosides (19), but did not interfere in this digitoxin FIA.

Digitoxin tracer preparations stored in methanol showed no significant change in their properties over at least one year. Anti-digitoxin solid-phase and digitoxin serum standards were stable for at least eight months. This stability of the reagents is an advantage for an assay that is requested infrequently in the United Kingdom, where digoxin is the cardiac glycoside of choice. In our laboratory, use of a radioimmunoassay involving \( ^{125}I \) was wasteful because not enough samples were assayed to utilize more than a fraction of the tracer before it had passed its expiration date.

Automation of the assay should be feasible, with use of the same procedures as developed for the continuous-flow automation of radioimmunoassay with magnetic separation (20), with the substitution of appropriate fluorometric endpoint detection.

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**References**