Improved Receptor Assay for Measuring Digoxin Activity

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We describe a receptor assay of digoxin activity involving Na+/K+-ATPase (EC 3.6.1.37) derived from human heart tissue. The procedure requires 500 μL of serum or plasma and incorporates one purification step, with Sep-Pak C18 cartridges (Waters). The method appears to be considerably more specific for digoxin and its cardio-active metabolites than are conventional immunoassays. Cardio-inactive metabolites and several digoxin-like factors cross react only slightly. The between-day coefficient of variation for the procedure ranged from 8.10% to 1.37% for digoxin concentrations between 0.5 and 5.0 nmol/L.

Additional Keyphrases: Na+/K+-ATPase • enzymic methods • heart tissues • monitoring therapy

Digoxin has been used for over 200 years to treat heart failure and cardiac arrhythmias (1-4). In spite of this, digoxin still does not meet many of the criteria currently used for therapeutic drug monitoring. For example, there still is no reliable method for its assay, and the correlation between concentration in serum and drug efficacy or toxicity is notoriously poor (5-8).

Digoxin appears to be fairly extensively metabolized in about two-thirds of the individuals who receive this drug (9). Many of the metabolites are cardio-active, others are cardio-inactive. Immunoassays of digoxin lack specificity and cross react with digoxin metabolites and so-called digoxin-like factors (10-19).

Several interesting correlations have been noted regarding the binding of cardiac glycosides to cardiac membranes and their positive inotropic effects (20-22). For example, there is a close relationship between inhibition of [3H]ouabain-receptor binding and inhibition of Na+/K+-ATPase (EC 3.6.1.37) activity. Several investigators have found that the concentration of cardiac glycosides that inhibited [3H]ouabain-receptor binding by 50% also inhibited Na+/K+-ATPase activity by 50% (23, 24).

We recently developed a receptor assay for measuring digoxin activity in an attempt to overcome some of the problems associated with therapeutic drug monitoring of this cardiac glycoside (19). The receptor utilized in that study was Na+/K+-ATPase from dog kidney. Although that method had attractive features, it still lacked both specificity and sensitivity; e.g., blank values for patients not receiving digoxin were usually substantial: ±1.8 nmol of digoxin equivalents per liter of blood. Generally, results for patients who were receiving the drug were considerably higher than those observed by fluorescence polarization immunoassay (19). Furthermore, between-day precision (CV) was poor, ranging between 21% and 10% for samples in the digoxin concentration range of 2.0 to 5.0 nmol/L.

Here we describe a greatly improved receptor assay for digoxin, in which Na+/K+-ATPase from human heart tissue is the receptor.

Materials and Methods

Materials

[3H]Ouabain (specific activity 22.1 kCi/mol) and [3H]digoxin (specific activity 13 kCi/mol) were obtained from New England Nuclear, Boston, MA.

From Sigma Chemical Co., St. Louis, MO, we obtained digoxin, dihydrodigoxin, dihydrodiogxin, bis-digioxtioisde, monodigioxtioisde, digoxigenin, digitoxose, and the following fatty acids: 20:4 arachidonic, 16:1 cis-palmitoleic, 18:1 oleic, 18:2 cis-linoleic, 18:2 trans-linolealaidic, 18:3 linolenic, 1-monolinoleoyl monoglyceride 18:2. The following steroids were also from Sigma: progesterone, cortisone, and 11α-hydroxyprogesterone, as were ATP (vanadium free) and ATPase (Grade IV, ouabain-sensitive, from dog kidney).

Whatman GF/B filters and other chemicals were from Fisher Scientific, Toronto, Canada. The supplier of complete Phase Combining System (PCS) scintillant was Amersham Corp. We used an LKB scintillation counter (Model 1217, Rackbeta; Fisher Scientific). The Sep-Pak C18 cartridges were from Waters Associates, Milford, MA.

Solutions

Preparation of digoxin standard samples in serum. Digoxin standards in plasma were prepared from the stock 1 mmol/L standard in methanol, to give concentrations of 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 15 nmol/L.

Metabolites of digoxin. We made up, separately, 10 mmol/L methanolic stock solutions of the six metabolites of digoxin. Each was then diluted 10-fold in buffer or plasma to give a 10 nmol/L concentration.

Unsaturated fatty acids were dissolved in methanol to give a stock solution, concentration 1 mmol/L. Each was then diluted in buffer or plasma to give working concentrations of 100, 150, or 200 μmol/L.

Steroids. We prepared initial stock solutions of progesterone, cortisone, and 11α-hydroxyprogesterone (1 mmol of each per liter) in absolute ethanol. Sequential 10-fold dilutions of each steroid were made in buffer or plasma to give concentrations of 10 nmol/L to 100 μmol/L.

Procedures

Preparation and purification of Na+/K+-ATPase from porcine and human heart tissue. All preparative procedures were carried out at 4 °C. Human hearts were removed at autopsy (<18 h post mortem). Fresh cardiac tissue was then dissected into variable structures (left atrium, left ventricle, right atrium, right ventricle), which we quick-froze in liquid nitrogen and stored at -70°C until used. Porcine heart from a local abattoir was treated as described for the human.

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heart tissue. We then thawed 500 g of frozen heart segment in 250 mL of homogenization medium (per liter, 0.32 mol of sucrose and 50 mmol each of Tris and EDTA, pH 7.4), minced this with scissors, and homogenized it in five volumes of homogenization medium, using a Polytron homogenizer at two-thirds of maximum speed for approximately 1 min. After filtering the suspension through two layers of cheesecloth, we homogenized the solution with a glass homogenizer and a Teflon pestle (five strokes, 20 s per stroke), then centrifuged the homogenate at 20,000 \times g for 40 min. After re-homogenizing the precipitate with sucrose buffer, we centrifuged it at 4000 \times g for 15 min. The combined supernates from three such centrifugations were further centrifuged at 40,000 \times g for 30 min. The resulting pellets were washed twice by resuspension with mild homogenization in a glass homogenizer, in ice-cold Tris buffer (50 mmol/L, pH 7.4, containing EDTA 0.5 mmol/L, NaCl 80 mmol/L, and MgSO_4 \cdot 7H_2O 4 mmol/L) and centrifuged at 40,000 \times g for 90 min.

Each pellet was suspended by gentle homogenization in 0.5 mL of Tris buffer, to give a final protein concentration of approximately 6 mg/mL. We quick-froze this suspension at -70 °C and used it for as long as two weeks. Protein was measured by the method of Lowry et al. (25), with bovine serum albumin as the standard. The yield was approximately 16 mg of protein per 100 g of fresh tissue.

*Inhibition of {[3H]}ouabain or {[3H]}digoxin binding to dog kidney, porcine heart, and human heart membrane.* We used a modification of a method described by Lichtstein and Samuelov (26). The diluent buffer was composed of Tris HCl (50 mmol/L, pH 7.4 at 37 °C), EDTA (disodium salt) 0.5 mmol/L, NaCl 80 mmol/L, and MgSO_4 \cdot 7H_2O 4 mmol/L. The reaction mixture consisted of 200 \mu L of digoxin standard extract or sample extract, 100 \mu L of ATP solution (5 mmol/L), and 100 \mu L of either dog-kidney ATPase suspension (approximately 250–700 U/L or 0.2–0.6 g of protein per liter) or porcine heart and human heart ATPase (0.16–0.6 g of protein per liter), incubated for 2 h at 37 °C in a water bath. After adding 100 \mu L of {[3H]}digoxin (0.150 mCi/L), we incubated the reaction mixture for another hour at 37 °C in a water bath, then terminated the reaction by placing the tubes in an ice bath for 5 min. Bound labeled digoxin was collected under suction by rapid filtration through the Whatman glass-filter membranes to separate free drug from membrane-bound drug. We washed the filters twice with 1.5 mL of cold buffer, then placed them in a scintillation vial. We added 10 mL of PCS scintillant to the vial, incubated at room temperature overnight, and counted the radioactivity in an LKB liquid scintillation counter. The standard curve ranged from 0 to 6 mmol/L for routine measurements of digoxin activity by the receptor assay. Specific studies as shown below in Figures 1 and 2 were performed with use of a standard curve that ranged from 0 to 15 nmol of digoxin per liter.

*Analyses of serum samples.* Serum or plasma from patients, or standards, were frozen and stored at -20 °C until analysis. We used a fresh Sep-Pak cartridge to extract each sample. Initially the Sep-Pak was activated with 20 mL of methanol, then washed with 20 mL of doubly distilled water before 500 \mu L of sample was applied to the cartridge under suction. The cartridge was then washed with 5 mL of distilled water, and 2 mL of methanol was added to elute the sample. We centrifuged the eluted sample for 2 min at 3000 \times g, then dried the methanolic supernate under N_2 at 40 °C in the fume hood. We reconstituted each sample in 300 \mu L of the Tris diluent buffer, and used 200 \mu L of this solution in the receptor assay.

**Results and Discussion**

*Sensitivity.* We encountered problems with poor sensitivity in our previous procedure (19), in which we used the dog-kidney preparation of ATPase. These problems have been largely overcome by changing the source of the receptor. As Figure 1 (top) shows, the binding of {[3H]}digoxin to receptor was greatest with the human-heart preparation, indicating that it has a greater concentration of receptor binding sites.
than do the other preparations. The source from which the receptors were isolated was important: in general, the efficacy of the receptor preparation decreased from human heart (right or left atrium or ventricle) to porcine heart to dog kidney.

Porcine heart Na⁺/K⁺-ATPase showed a higher affinity, as expressed by the inhibitory concentration at 50% (IC50), than dog kidney Na⁺/K⁺-ATPase (9.5 vs 10.0 nmol/L). Human heart Na⁺/K⁺-ATPase showed the highest affinity of the three preparations, with IC50 values of 7.5 nmol/L in the left or right ventricle and 7.0 nmol/L in the left atrium.

Figure 1 (bottom) illustrates that the percentage displacement of [³H]digoxin from the receptor by unlabeled digoxin was considerably greater for the human-heart preparation than for receptor prepared from other sources; e.g., at 15 nmol/L, digoxin displaced 23% more labeled digoxin from receptor isolated from the left atrium of human heart than from receptor isolated from dog kidney.

**Effect of protein concentration.** Figure 2 shows the effect of digoxin concentration on displacement of the labeled drug from the receptor at different receptor concentrations. Greatest sensitivity was obtained with a protein concentration of 0.16 g per liter of receptor preparation. However, the figure for total counts per minute with such a concentration of receptor preparation is very low (about 1000), which does not permit good reproducibility. Lower protein concentrations were not tested. Optimal sensitivity and reproducibility were obtained by using receptor preparations (from human heart) containing protein concentrations from 0.5 to 0.9 g/L, which provided about 6000 counts/min.

**Precision.** The between-day precision (Table 1) is considerably better in the procedure involving the heart receptor than that previously reported for the dog-kidney preparation (19).

**Background readings obtained with the receptor assay.** Serum was sampled from 11 normal volunteers who were receiving neither cardiac glycosides nor other medications. Using the heart receptor preparation, we obtained background concentrations of <0.4 nmol of digoxin equivalents per liter in all cases. In comparison, the dog-kidney receptor preparation in a similar study yielded background readings of up to 2.0 nmol/L (19).

**Comparisons with patients’ samples.** Comparing results obtained by the receptor assay with those obtained by fluorescence polarization immunoassay (FPIA; Abbott Digoxin Protocol 1) yielded a regression equation y(RRA) = 0.504x (FPIA) + 0.248 and a correlation coefficient (r) of 0.5824 (Figure 3). Mean results (digoxin equivalents) for 48 samples by the receptor assay were somewhat lower than those by the FPIA technique, 0.81 vs 1.13 nmol/L. Studies to evaluate which procedure provides the most clinically meaningful results are currently underway.

| Table 1. Between-Day Precision of the Receptor Assay for Digoxin |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Digoxin concn, nmol/L       | 0.5                        | 1.5                        | 3.0                        | 5.0                        |
| n                           | 10                         | 9                          | 9                          | 11                          |
| Mean, nmol/L                | 0.49                       | 1.35                       | 3.1                        | 5.1                        |
| SD, nmol/L                  | 0.04                       | 0.07                       | 0.12                       | 0.07                       |
| CV, %                       | 8.10                       | 5.18                       | 3.87                       | 1.37                       |

Receptor preparation from human heart ventricle tissue.
Analyses of proficiency-testing samples. A proficiency-testing sample distributed by the American Society of Clinical Pathologists (CP86-3, 1986) and containing digoxin (2.56 nmol/L), digitoxin (5.12 nmol/L), 11α-hydroxyprogesterone (0.05 mg/L), progesterone (1.0 mg/L), and cortisone (1.0 mg/L) was analyzed for digoxin by radioimmunoassay, FPIA, and the receptor assay. The respective results were 7.4, 9.4, and 2.9 nmol/L. The most nearly accurate result was provided by the receptor assay; both immunoassays showed poor specificity.

Interference by steroids, fatty acids, and digoxin metabolites. The cardio-inactive metabolites of digoxin do not cross react in the Na+/K+-ATPase receptor assay from human-heart membrane or from dog-kidney membrane. The cardio-active metabolites of digoxin are detected by receptor assay in an amount approximately equal to their respective cardio-activities (Table 2), in contrast to their behavior in the FPIA (19).

Unsaturated fatty acids cross react in immunoassays and receptor assays for digoxin (18, 19). Indeed, binding of [3H]digoxin to dog kidney Na+/K+-ATPase was completely inhibited by arachidonic acid (150 μmol/L) or linolealaidic acid (200 μmol/L), whereas these concentrations did not completely inhibit binding in experiments with human-heart receptor (Table 3). Some displacement of [3H]digoxin was demonstrated by oleic, linoleic, linolealaidic, and arachidonic acids at 100 μmol/L in dog kidney Na+/K+-ATPase, but these effects were less when the receptor was of human heart origin.

Potential interference from the steroids progesterone, cortisone, and 11α-hydroxyprogesterone was tested at four concentrations (Table 4). Progesterone, which is slightly cardio-active, cross reacts minimally in both dog-kidney and human-heart receptor assay at 10 μmol/L. The cardio-active steroids cortisone and 11α-hydroxyprogesterone do not cross react in either of the receptor preparations at 10 μmol/L. In contrast, all three steroids cross react significantly in the FPIA procedure at 10 μmol/L.

Interference by digoxin-like factors. It has previously been reported that digoxin measurements can be falsely increased in cord sera (27), in sera from patients with kidney or liver failure, and in sera obtained in the third trimester of pregnancy (15). Table 5 shows that, although digoxin-like factors interfere appreciably in the FPIA procedures evaluated, they generated considerably less interference in the human-heart receptor assay.

In conclusion: The receptor assay developed with the human-heart receptor is both more specific and sensitive than that previously reported by our group involving the dog-kidney preparation. The assay now affords good precision and would appear to have many advantages over conventional immunoassay procedures, e.g., lack of interference by steroids (Table 5) and relatively cardio-inactive metabolites (Table 3). Clinical studies to evaluate the correlation between efficacy and toxicity and results obtained by both the receptor assay and FPIA technique are currently underway.
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References