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# Radioimmunoassay of Digoxin in Serum Using Monoclonal Antibodies and Assessment of Interference by Digoxin-Like Immunoreactive Substances

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Summary: We used 7 monoclonal antibodies (MoAbs) and one polyclonal antibody to develop radioimmunoassays (RIAs) for digoxin in serum or plasma. These RIAs were tested for measuring apparent digoxin concentrations in serum from patients receiving the drug, from normal individuals, and in cord blood plasma. We found that two MoAbs cross-reacted significantly with substances in cord blood. The magnitude of cross-reactivity was dependent on the incubation time and temperature. Under equilibrium conditions, one antibody gave apparent digoxin values in cord blood plasma averaging 2.15 ng/ml. We suggest that this cross-reactivity is partially due to progesterone and 17-hydroxyprogesterone in cord blood plasma. The antibody that shows high cross-reactivity with digoxin-like immunoreactive substances may prove a useful tool for studies dealing with characterization of the cross-reacting compounds. **Key Words:** Digoxin—Radioimmunoassay—Monoclonal antibodies—Digoxin-like immunoreactive substances—Cross-reactivity.

Certain groups of patients have in their serum substances that cross-react with digoxin antibodies (1,2). These substances are now referred to in the literature as digoxin-like immunoreactive substances (DLIS). DLIS are known to be present at high concentration in neonatal (3–6) and maternal serum, in amniotic fluid (3,7,8), in patients with renal (9) or hepatic (10) failure, and in cord blood plasma (11–13).

Many commercial digoxin kits available today are based on polyclonal antibodies. Although monoclonal antibodies (MoAbs) for digoxin have been successfully produced (14,15), they have rarely been applied for digoxin determination in serum (16), because polyclonals are very well established and the early MoAbs for digoxin were characterized by low affinity. In addition, the specificities of MoAbs are different and, as shown previously (14,15), different clones show specificities for certain areas of the digoxin molecule.

To decrease the DLIS cross-reactivity of commercial kits for digoxin, many investigators introduced a number of modifications with partial success (3,6,8,13,17,18). In this study, we examined the suitability of seven different MoAbs for digoxin for devising radioimmunoassays (RIAs) for the drug. We also included one well-established polyclonal antibody for comparison. We examined the DLIS

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cross-reactivity in detail because we wished to isolate the most specific digoxin RIA system and a system more sensitive to DLIS. The latter could be a valuable tool in studying further the nature and physiologic role of DLIS present in serum. Cross-reactivity with several steroids was also included because many studies suggest that some DLIS are steroids (11,19–21).

## MATERIALS AND METHODS

### Specimen

We collected sera from 49 normal adult subjects not taking digoxin or any other medication. We also prepared 10 cord blood plasma pools (CBPs) as described previously (11,13). Another 21 sera were collected from patients receiving digoxin. All samples were stored at −20°C until use.

### Antibodies

The polyclonal antidigoxin antiserum (rabbit) was obtained from Western Chemical Research Corporation, Ft. Collins, CO, U.S.A. (antibody H). The MoAbs used were obtained from commercial sources or investigators (Table 1).

### Digoxin Label

We examined three different commercial preparations of $^{125}$I-labeled digoxin. A [$^{125}$I]tyrosine derivative of digoxin from Diagnostic Products, Los Angeles, CA, U.S.A. (specific activity 0.03 μCi/ml) was rejected because of poor antibody binding. A [$^{125}$I]histamine derivative of digoxin from New England Nuclear, North Billerica, MA, U.S.A. was rejected because of its low specific activity (0.04 μCi/ml). We used a [$^{125}$I]histamine derivative of digoxin from Amersham International, U.K., throughout this study (specific activity 0.45 μCi/ml).

### Antibody Dilution

We empirically calculated the antibody dilution of all antibodies used. The working dilution was the one that would bind 50% of 40 nCi [$^{125}$I]digoxin under the conditions used for the assay and was A, $10^4$; B, $2 \times 10^4$; C, $1.6 \times 10^4$; D, $1.5 \times 10^4$; E, $1.2 \times 10^4$; F, $1.6 \times 10^4$; G, $9 \times 10^4$; and H, $3 \times 10^5$ (see Table 1 for antibody identification; antibody H is the polyclonal). Antibodies were diluted in a 50 m*M* phosphate buffer, pH 7.5, containing 5 g bovine serum albumin and EDTA 0.13 m*M*. Free and bound [$^{125}$I]digoxin was separated by a second antibody technique (described below). Incubation times were optimized as described later.

### Cross-Reactivity Studies

All steroids used in this study (Table 2) were purchased from Serva Biochemical, Heidelberg, F.R.G. except for DHEA-$SO_4$ (Sigma Chemical, St. Louis, MO, U.S.A.). The solution of the steroids investigated were prepared in digoxin-free normal plasma at concentrations normally seen in human cord plasma at term. In these samples, digoxin im-

**TABLE 1.** *Monoclonal anti-digoxin antibodies used in this study*

| Monoclonal antibody | Source | Code | Purity | Affinity[a] $M^{-1}$ |
|---|---|---|---|---|
| A | Chemicon[b] | MAB 515 | Ascites | — |
| B | Medix[c] | 133-01 | DEAE[d] | $1 \times 10^9$ |
| C | Medix | 133-02 | DEAE[d] | $2 \times 10^9$ |
| D | Medix | 133-03 | DEAE[d] | $2.5 \times 10^9$ |
| E | Scandibodies[e] | — | Ascites | — |
| F | MGH[f] | 45-20 | Ascites | $1.7 \times 10^{12}$ |
| G | MGH | 40-160 | Ascites | $2.6 \times 10^{11}$ |

DEAE, diethylaminoethanol; MGH, Massachusetts General Hospital.
[a] Calculated by the developers of the antibodies.
[b] Chemicon International, El Segundo, CA, U.S.A. 90245.
[c] Medix Biotech, Foster City, CA, U.S.A. 94404.
[d] Ascites purified by DEAE column chromatography before use.
[e] Scandibodies Laboratory, Santee, CA, U.S.A. 92071.
[f] Antibodies were provided by Dr. Meredith Mudgett-Hunter, MGH; they are further described in refs. 14 and 15.

TABLE 2. *Cross-reactivity of various steroids with antibody F at 4°C*

| Steroid[a] | Concentration[b] (ng/ml) | Cross-reactivity (ng/ml digoxin equivalents) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Incubation time (h) | | | | | |
| | | 0.5 | 1 | 4 | 8 | 24 | 96 |
| Aldosterone | 0.8 | ND[c] | ND | ND | ND | ND | ND |
| Cortisol | 100 | 0.36 | ND | ND | ND | ND | ND |
| DHEA-S | 3,000 | 0.32 | ND | ND | ND | ND | ND |
| Estradiol | 10 | ND | ND | ND | ND | ND | ND |
| Progesterone | 300 | 0.95 | 0.65 | 0.40 | 0.37 | 0.36[d] | 0.37 |
| 17-hydroxyprogesterone | 33 | 0.90 | 0.66 | 0.44 | 0.41 | 0.42[d] | 0.40 |
| Testosterone | 7 | ND | ND | ND | ND | ND | ND |

[a] All steroids for this experiment were dissolved in digoxin-free normal plasma.
[b] Steroids were tested at levels similar to those found in cord blood plasma.
[c] Cross-reactivity not detected.
[d] Cross-reactivity of progesterone and 17-hydroxyprogesterone at 18°C after 24-h incubation was 0.64 and 0.63 ng/ml digoxin equivalents, respectively.

munoreactivity was estimated at different incubation times and temperatures (Table 2). Cross-reactivity was expressed as digoxin equivalents in nanograms per milliliter (Table 2).

## General RIA Procedure

One hundred microliters standard digoxin solutions or samples were pipetted in tubes, and 100 μl [$^{125}$I]digoxin tracer was added. Another 100 μl antibody was then added (diluted as described above), and the tubes were briefly mixed by vortexing. After 24-h incubation at 18°C (equilibrium conditions), 100 μl donkey anti-rabbit antiserum (for antibody H, diluted 1:20, Wellcome Diagnostics, Dartford, England) or rabbit anti-mouse antiserum (for antibodies A–G, diluted 1:15, Dakopatts, Glostrup, Denmark) was added. After 30-min incubation at 18°C, 1 ml ice-cold polyethylene glycol solution (14% wt/vol; in 9 g/L NaCl solution) (PEG 6000; Serva) was added in all tubes. The tubes were then centrifuged at 2,500 g for 30 min at 4°C. The supernatant was discarded by aspiration, and radioactivity was measured in the pellet. All measurements were performed in triplicate.

## RESULTS

The detection limit of all assays developed using antibodies A–H was <0.11 ng/ml. Sensitivity was calculated as the concentration corresponding to zero mean counts minus 3 SD. We measured digoxin in the serum of patients receiving the drug, with all eight assays developed and found reasonable agreement between results (Table 3). Table 4 summarizes the results obtained with the eight antibodies for 49 samples from normal individuals not receiving digoxin or any other medication. The mean digoxin equivalent concentration obtained was <0.10 ng/ml in all samples.

Figure 1 summarizes the results obtained by using the eight antibodies for 10 CBPs. Of the eight antibodies, only antibody C (mean 0.86 ± 0.20 ng/ml digoxin equivalents) and antibody F (mean 2.15 ± 0.32 ng/ml of digoxin equivalents) cross-reacted significantly with substances present in cord blood plasma. For this reason, we used only antibodies C, E (as control), and F for the subsequent studies.

The effect of both incubation time and temperature on the cross-reactivity of antibody F with DLIS present in CBPs was examined in detail (Table 5). Cross-reactivity decreased and stabilized within 8 h at 18°C and within 24 h at 4°C. At 18°C, the final value of DLIS was higher than that observed at 4°C.

To study the effect of dilution on the results obtained with antibody F, we serially diluted a CBP sample with the digoxin zero standard and reanalyzed it at either 4°, 18°, or 37°C, at incubation times at which cross-reactivity stabilizes (i.e., ≥24 h). The results are shown in Table 6. Dilution was not linear.

Steroid cross-reactivity studies were performed using antibodies C, E, and F. With antibody E, no measurable cross-reactivity was observed with the steroids shown in Table 2 at the concentrations and conditions tested. Only 17-hydroxyprogesterone cross-reacted significantly with antibody C. At a 17-hydroxyprogesterone concentration of 33 ng/ml, the cross-reactivity of antibody C was 1.90 ng/ml

TABLE 3. *Assay of digoxin in serum from patients receiving the drug with various antibodies*

| | Antibody (1) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Digoxin (ng/ml) | | | | | | | |
| Sample | A | B | C | D | E | F | G | H |
| 1 | 1.29 | 1.51 | 1.17 | 1.14 | 1.32 | 1.15 | 1.30 | 1.23 |
| 2 | 0.99 | 1.17 | 0.96 | 0.94 | 0.98 | 1.03 | 1.14 | 1.01 |
| 3 | 0.85 | 0.95 | 0.79 | 0.78 | 0.82 | 0.69 | 1.02 | 0.79 |
| 4 | 1.02 | 1.19 | 1.06 | 0.88 | 1.03 | 1.10 | 0.78 | 0.92 |
| 5 | 1.26 | 1.38 | 1.08 | 1.09 | 1.26 | 1.06 | 0.84 | 1.12 |
| 6 | 1.30 | 1.58 | 1.00 | 1.13 | 1.25 | 1.00 | 0.65 | 1.02 |
| 7 | 1.22 | 1.49 | 1.11 | 1.04 | 1.25 | 1.05 | 1.00 | 1.01 |
| 8 | 1.19 | 1.43 | 1.07 | 1.02 | 1.09 | 1.11 | 0.96 | 1.14 |
| 9 | 1.45 | 1.74 | 1.21 | 1.34 | 1.48 | 1.28 | 1.33 | 1.32 |
| 10 | 2.19 | 2.67 | 2.30 | 2.27 | 2.44 | 2.91 | 2.19 | 2.21 |
| 11 | 0.63 | 1.01 | 0.74 | 0.83 | 0.79 | 0.91 | 1.17 | 0.85 |
| 12 | 1.19 | 1.46 | 1.25 | 1.19 | 1.21 | 1.25 | 0.83 | 1.24 |
| 13 | 0.97 | 1.22 | 0.99 | 1.03 | 1.06 | 1.07 | 1.32 | 0.99 |
| 14 | 1.12 | 1.36 | 1.29 | 1.09 | 1.19 | 1.74 | 0.96 | 1.11 |
| 15 | 0.64 | 0.90 | 0.61 | 0.73 | 0.62 | 1.17 | 1.08 | 0.78 |
| 16 | 1.04 | 1.31 | 1.02 | 0.96 | 1.02 | 1.05 | 0.75 | 0.97 |
| 17 | 1.81 | 2.19 | 1.98 | 1.79 | 1.87 | 1.92 | 1.00 | 1.97 |
| 18 | 1.51 | 1.93 | 1.73 | 1.63 | 1.71 | 1.59 | 2.05 | 1.58 |
| 19 | 1.09 | 1.33 | 1.12 | 0.99 | 1.25 | 1.10 | 1.70 | 1.28 |
| 20 | 1.28 | 1.35 | 1.29 | 1.08 | 1.11 | 1.40 | 1.10 | 1.20 |
| 21 | 1.66 | 1.67 | 1.39 | 1.20 | 1.35 | 1.37 | 1.15 | 1.24 |

Antibody description is given in footnote to Table 1.

digoxin equivalents after 24-h incubation at 18°C. Antibody F cross-reacted significantly especially with progesterone and 17-hydroxyprogesterone (Table 2). Cross-reactivity decreased as the incubation time was prolonged but stabilized after ~8 h of incubation at 4°C. Cross-reactivity at 18°C was higher than the cross-reactivity observed at 4°C.

## DISCUSSION

In our previous study on DLIS present in CBPs (13), we verified that cross-reactivity is highly dependent on the antibody used (among three polyclonals) and decreases dramatically when incubation time is prolonged or when incubation temperature is increased. The decrease in cross-reactivity caused by increasing the temperature and incubation time is a general phenomenon observed for steroid immunoassays (22).

TABLE 4. *Digoxin equivalent concentration obtained for analysis of 49 serum samples from normal individuals*

| | Digoxin (ng/ml) | | | |
|---|---|---|---|---|
| Antibody[a] | Mean | SD | Minimum | Maximum |
| A | 0.03 | 0.07 | ND | 0.24 |
| B | 0.09 | 0.07 | ND | 0.26 |
| C | 0.04 | 0.03 | ND | 0.11 |
| D | 0.04 | 0.05 | ND | 0.16 |
| E | 0.03 | 0.06 | ND | 0.24 |
| F | 0.03 | 0.04 | ND | 0.15 |
| G | 0.08 | 0.06 | ND | 0.17 |
| H | 0.01 | 0.02 | ND | 0.07 |

ND, not detectable.
[a] Antibody description is given in legend to Table 1.

We examined one well-established polyclonal and seven MoAbs with respect to their cross-reactivity with DLIS present in CBPs. With the exception of two MoAbs (C and F), all other antibodies showed no cross-reactivity with DLIS present in cord blood plasma (Fig. 1). We preferred to use long incubation times during these studies to be able to measure cross-reactivity under equilibrium conditions. Under such conditions, we also verified that none of the antibodies produced significant cross-reactivities with sera from normal individuals (Table 3). Antibody C cross-reacted to a degree with DLIS present in CBPs. We concentrated on antibody F, which was well characterized previously (14,15). We verified that cross-reactivity decreases with an increase in incubation time and temperature and reaches a steady value that is slightly lower at 4°C than at 18°C (Table 5). These data are in agreement with those we reported recently with poly-

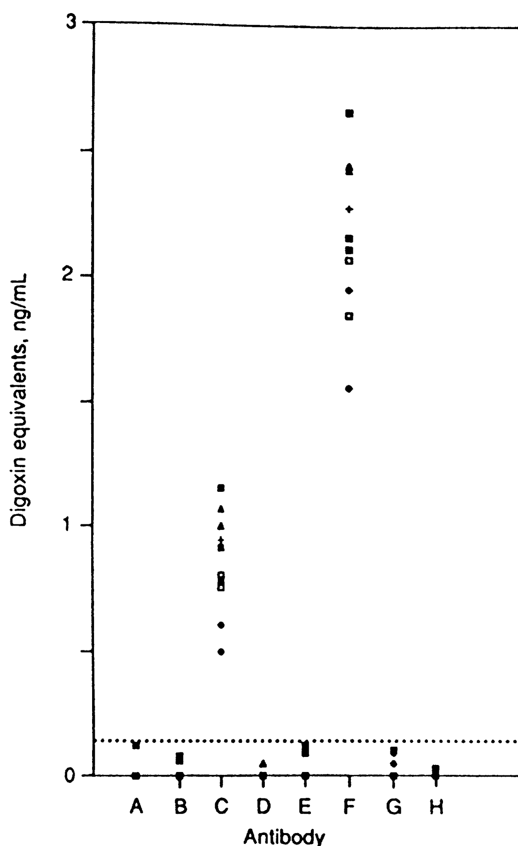


FIG. 1. Cross-reactivity (in digoxin equivalent concentration) of digoxin-like immunoreactive substances present in cord blood plasma samples with eight different antibodies described in Table 1 (details provided in text).

clonal antibodies (13). The non-parallelism observed after diluting a CBP sample (tested at three different temperatures) (Table 6), is an expected phenomenon that occurs when the substance to be measured is not a true analyte but a cross-reactant (23).

TABLE 6. *Diluton results for a cord blood plasma sample using antibody F*[a]

| Variable | Digoxin equivalents (ng/ml)[b] | | |
|---|---|---|---|
| Temperature (°C) | 4 | 18 | 37 |
| Undiluted | 1.55 ± 0.07 | 2.03 ± 0.05 | 2.16 ± 0.05 |
| 1/1.3 | 1.35 ± 0.04 | 1.64 ± 0.02 | 1.75 ± 0.03 |
| 1/2 | 1.04 ± 0.01 | 1.22 ± 0.03 | 1.14 ± 0.02 |
| 1/4 | 0.84 ± 0.03 | 0.78 ± 0.05 | 0.62 ± 0.04 |
| 1/8 | 0.61 ± 0.04 | 0.38 ± 0.05 | 0.33 ± 0.03 |

[a] Dilutions were made with the zero digoxin standard.
[b] Each temperature experiment has its own standard curve.

Antibodies F and G were characterized previously. Antibody G recognizes primarily determinants of the steroid portion of digoxin and to a lesser extent those present in the digitoxose sugar residues (14,15). This antibody does not discriminate between digoxin and digitoxin, but steroids like cholesterol, testosterone, 17-β-estradiol, progesterone, cortisol, and 4-androsterone do not cross-react.

Antibody F also cross-reacts very highly with digitoxin, indicating that the $C_{12}$-OH is not an important determinant. This antibody cross-reacts significantly with progesterone and 17-hydroxyprogesterone (Table 2) which are present at relatively high concentrations in cord blood plasma (24). Thus, progesterone and 17-hydroxyprogesterone could account for roughly half of the cross-reactivity observed in cord blood plasma by using antibody F. The remaining immunoreactivity may result from substances not yet identified.

The main conclusions of our studies are as follows. MoAbs for digoxin can be used successfully

TABLE 5. *Effect of incubation time and temperature on cross-reactivity of antibody F*

| CBP sample | Incubation temperature (°C) | Digoxin equivalents (ng/ml)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Incubation time (h) | | | | | |
| | | 0.5 | 1 | 4 | 8 | 24 | 96 |
| 1 | 4 | 4.25 | 3.80 | 2.85 | 2.09 | 1.41 | 1.39 |
| | 18 | 4.62 | 2.81 | 1.96 | 1.78 | 1.75 | 1.75 |
| 2 | 4 | 5.16 | 4.60 | 3.60 | 2.58 | 1.73 | 1.72 |
| | 18 | 5.16 | 3.72 | 2.28 | 2.08 | 2.06 | 2.01 |
| 3 | 4 | 5.16 | 4.40 | 3.10 | 2.22 | 1.53 | 1.54 |
| | 18 | 5.16 | 3.20 | 2.04 | 1.98 | 2.01 | 1.96 |
| 4 | 4 | 4.96 | 4.00 | 2.86 | 2.21 | 1.50 | 1.51 |
| | 18 | 4.54 | 2.92 | 1.95 | 1.85 | 1.85 | 1.83 |
| 5 | 4 | 5.16 | 4.50 | 3.06 | 2.47 | 1.64 | 1.63 |
| | 18 | 5.16 | 3.01 | 2.09 | 1.90 | 1.92 | 1.91 |

CBP, cord blood plasma.
[a] Each time/temperature experiment has its own standard curve.

for measurement of the drug in serum provided that they are carefully assessed in terms of specificity. Of the seven MoAbs tested, five did not cross-react with substances present in cord blood plasma. Two MoAbs cross-reacted significantly, yielding readings for digoxin averaging 0.86 ng/ml (antibody C) and 2.15 ng/ml (antibody F) in cord blood plasma, under equilibrium conditions. Cross-reactivity can result in readings as high as 5 ng/ml with antibody F if short incubation times are used (Table 2). We showed that antibody F cross-reacts only with progesterone and 17-hydroxyprogesterone at equilibrium conditions for DLIS (Table 2). We also propose for the first time that antibody F may be a useful tool in studying DLIS present during embryonal and neonatal life because of its high cross-reactivity with DLIS. To our knowledge, this antibody can produce the highest apparent digoxin readings when CBP is used as a sample. We are now using the same antibody to identify DLIS in other clinical situations, and we purify DLIS present in cord blood plasma using this antibody as immunoextraction reagent.

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