The present method provides several practical advantages rendering it suitable for routine application in the clinical laboratory. The use of enzyme as a label instead of a radioisotope eliminates the disadvantages associated with RIAs. By coating the microtitre plate first with low-cost second antibody, the use of specific anti-DHEA-S at high dilutions is possible. With the one-step technique, total incubation time is reduced to only 2 h, compared with 3 h for the two-step technique. Furthermore, this microtitre plate technique is much more practicable than the classical coated-tube EIAs. By the MP-EIA more than 200 samples can be handled by one technician per working day. The very low sample requirement facilitates measurement of DHEA-S, even in the newborn.

In contrast to current MP-EIA techniques, a single plate, once coated with second antibody, may be used for the simultaneous immunoassay of other compounds, provided the corresponding immunoassay reagents are used. This facility makes the present version of MP-EIA very economical. We think that the principle of this method is more widely applicable in the field of antigen estimation by enzyme immunoassay.

Financial support for T. K. D. from the Deutscher Akademischer Austauschdienst (DAAD) is gratefully acknowledged. We thank Mrs. E. Müllen for excellent secretarial assistance.

References

Fluorescence Energy Transfer Immunoassay of Digoxin in Serum
Mario Plebani and Angelo Burlina

We have evaluated the reliability of the new fluorescence energy transfer immunoassay (FETI) for determining concentrations of digoxin in serum. The method demonstrated good stability of the standard curve and satisfactory precision in both within- and between-assays. We have also found a significant correlation between FETI and two RIA methods (r = 0.97). Furthermore, the association between results by FETI and by the EMI method (a conventional enzyme immunoassay) was a linear one. Finally, the assay encountered no significant interference from endogenous substances (hemoglobin, bilirubin, and triglycerides).

Digoxin is the most widely prescribed cardiac glycoside for the treatment of congestive heart failure and for the management of supraventricular tachyarrhythmias (1). However, its narrow therapeutic index necessitates individualized adjustment of dosage to achieve optimal response.

The value of monitoring digoxin concentrations in the clinical management of patients being treated with this drug is now well established (2). In most clinical laboratories, the technique of choice is radioimmunoassay (RIA). However, despite its good specificity and sensitivity, RIA has the well-known disadvantages associated with isotopic techniques (radioactivity, special licensing requirements, short lifetimes of the labels). Furthermore, in some clinical situations RIA methods for determining the concentrations of digoxin are not sufficiently rapid.

Recently, some radioactive immunological methods have been replaced with nonisotopic techniques. Here we report our evaluation of a new method for quantifying digoxin, based on the principle of fluorescence energy transfer.

Originally described by Forster (3), the principle of fluorescent energy transfer is based on the transfer of energy from an electronically excited fluorescent dye to a fluorescent acceptor dye. The fluorescence decay of the electronically excited molecule is associated with a change in the electric dipole moment of the molecule. In the proximity of a second molecule, energy is transferred through dipole–dipole coupling. Such a transfer will lead to quenching of the fluorescence of the donor and cause the acceptor to fluoresce (4, 5). Little quenching results when there is a high concentration of analyte, but a high proportion of the fluorescence is quenched when a low concentration of analyte is present.

CLIN. CHEM. 31/11, 1879–1881 (1985)

Mario Plebani and Angelo Burlina

Laboratory of Clinical Chemistry and Microscopy, I-35128 Padova, Italy.

Received May 10, 1985; accepted July 30, 1985.
Materials and Methods

Samples. Serum was collected from 100 patients (both hospitalized and outpatients) who were being treated with digoxin. The specimens were divided and aliquots were either assayed the same day by fluorescence energy transfer immunosay (FETI), EMTR (a conventional enzyme immunoassay; trademark of Syva Co., Palo Alto, CA) and fluorescence polarization immunosay (FPIA), or stored at -20 °C until analyzed by RIA.2

Control sera (Lymphochek, lot no. 7900; and Bi-Level Cardiac Drug, lot nos. 72YO1 and 73Y02) were purchased from Bio-Rad Laboratories, Segrate, Italy, and from Ortho Diagnostic Systems SpA, Milano, Italy, respectively. Digoxin was obtained from Sigma Chemical Co., St. Louis, MO 63178.

FETI. The reagents for FETI (Syva Co., Palo Alto, CA 94304) were obtained from Bracco Ind. Chimica, Milano, Italy. The fluorescence probe used in this homogeneous assay is β-phycocerythrin, a protein from red algae; its excitation peak is 545 nm and emission peak 575 nm. The binding of the fluorescer-labeled digoxin to the quencher-labeled antibody decreases the fluorescence signal.

Apparatus. The assay was performed with a Syva Advance System, an automated fluorometric analyzer designed specifically for use with FETI Syva Advance Assays and also for EMTR assays for therapeutic drugs. The instrument consists of two principal subunits, a pipetter unit for reagent handling and a fluorometer unit where samples are introduced and assayed. The instruments are controlled by an internal microprocessor system that monitors and coordinates all functions.

A lot-specific Advance Program Card is used to program the microprocessor with more than 30 assay-specific parameters, including the correct instrument settings for sample and reagent volume, flow-cell temperature, and the timing of the reaction measurement. The card also specifies the assay range, concentration units, calibration parameters, and quality-control limits.

Procedure. To destroy interfering proteins, we incubate 200 µL of each calibrator, control, or sample with 800 µL of an oxidizing agent. The time during which the sample reacts with this agent is critical and should be between 5 and 20 min; if longer than this, the test does not give reliable results.

No separation step is required because sodium sulfite in the assay buffer neutralizes the oxidizer. Therefore, after 5 to 20 min, the analyzer adds the reagents (fluorescer-labeled digoxin and quencher-labeled antibody) and makes the first reading of fluorescence. The system then "waits" 21 min before taking a second fluorescence reading for each sample, and calculating corresponding rate for each. The drug concentration in each sample is determined by comparison with the calibration curve.

Correlation studies. Using linear regression analysis, we statistically compared the results of the FETI method with those by two RIA methods and also with those by EMTR method. RIA kits were from commercial sources as follows: Digoxin Micromedic (Miles Italiana SpA, Cavenago Brianza, Italy) and Digoxin MAIA (Biodata, Milano, Italy). The EMTR assay was performed with the reagents from Syva Co. and with a Multistat III microcentrifugal analyzer (Instrumentation Laboratory SpA, Milano, Italy). For FPIA we used the TDX analyzer and its specifically designed reagents (Abbott Divisione Diagnostici, Roma, Italy).

Results

Standard curve stability. We evaluated the stability of the standard curve by assaying five patients' samples and three control sera at 0, 6, 24, 48, 96, and 120 h and comparing the results obtained at the different time intervals. As shown in Table 1, the standard curve is stable for at least 120 h. In fact, the results obtained for samples by comparison with a calibration curve set up 120 h earlier are very similar to those obtained by assaying the calibrators and the sera in the same batch.

Precision. To determine the within-run and between-run precision of the assay, we assayed five serum pools containing different concentrations of digoxin. For within-run precision, each sample was assayed 21 times; for between-run precision, each sample was assayed 11 times on 11 different carousels. As Table 2 shows, the reproducibility of the test is satisfactory at all five concentrations analyzed. The high CV for the serum sample containing 0.4 µg of digoxin per liter is to be expected, considering the low concentration being measured.

Accuracy. We performed analytical recovery tests to evaluate the accuracy of the FETI method. A set of sera supplemented to contain 0.8, 1.6, 2.4, 3.2, and 6.4 µg of digoxin per liter were added to equal volumes of human serum (digoxin content, 0.98 µg/L), then analyzed by FETI. The results obtained were very close to the expected values, the mean analytical recovery being 98.4% (range, 96 to 100%). Similar results were obtained by adding the same concentrations of digoxin to two other sera with low or high protein concentration (25 and 83 g/L, respectively).

Linearity. The range of linearity of the assay was assessed by diluting a serum containing a high concentration of digoxin with a serum that was digoxin-free. The digoxin concentrations measured were very close to expected values; the test, therefore, shows a good dilution recovery (see Figure 1).

Table 1. Digoxin Results (µg/L) Obtained with the Same Standard Curve

<table>
<thead>
<tr>
<th>Patients' sera</th>
<th>Time, h</th>
<th>0</th>
<th>6</th>
<th>24</th>
<th>48</th>
<th>96</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td></td>
<td>0.37</td>
<td>0.36</td>
<td>0.38</td>
<td>0.36</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>MP</td>
<td></td>
<td>1.78</td>
<td>1.70</td>
<td>1.83</td>
<td>1.72</td>
<td>1.90</td>
<td>1.77</td>
</tr>
<tr>
<td>CM</td>
<td></td>
<td>1.44</td>
<td>1.34</td>
<td>1.37</td>
<td>1.32</td>
<td>1.39</td>
<td>1.30</td>
</tr>
<tr>
<td>SB</td>
<td></td>
<td>1.00</td>
<td>1.09</td>
<td>1.14</td>
<td>1.13</td>
<td>1.17</td>
<td>1.14</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C I</td>
<td></td>
<td>0.74</td>
<td>0.65</td>
<td>0.76</td>
<td>0.72</td>
<td>0.77</td>
<td>0.78</td>
</tr>
<tr>
<td>C II</td>
<td></td>
<td>1.79</td>
<td>1.71</td>
<td>1.80</td>
<td>1.76</td>
<td>1.74</td>
<td>1.74</td>
</tr>
<tr>
<td>C III</td>
<td></td>
<td>2.57</td>
<td>2.45</td>
<td>2.48</td>
<td>2.50</td>
<td>2.60</td>
<td>2.65</td>
</tr>
</tbody>
</table>

All aliquots of the same samples were assayed at the times indicated.

Table 2. Precision of the FETI Method

<table>
<thead>
<tr>
<th>Digoxin, µg/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within run (n = 21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>0.04</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>1.75</td>
<td>0.06</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>2.02</td>
<td>0.05</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>2.84</td>
<td>0.06</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.08</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Between run (n = 11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>0.03</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>1.74</td>
<td>0.07</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>2.01</td>
<td>0.04</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>2.9</td>
<td>0.07</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>3.35</td>
<td>0.09</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

Nonstandard abbreviations: FETI, fluorescence energy transfer immunosay; EMTR, enzyme multiplied immunosay; FPIA, fluorescence polarization immunosay.3

3 Nonstandard abbreviations: FETI, fluorescence energy transfer immunosay; EMTR, enzyme multiplied immunosay; FPIA, fluorescence polarization immunosay.
Fig. 1. Linearity of the FETI method for digoxin concentrations in serum at various dilutions (mean ± SD of three determinations)

Abscissa, expected values; ordinate, measured values

Correlation studies. Results from FETI (y) and the first RIA method (Digoxin Micromedic) (x) correlated well (r = 0.967, slope 0.925, intercept +0.124, S,

y = 0.161, n = 60). Similar results were obtained with FETI and the second RIA method (Digoxin MAIA): the correlation coefficient was 0.97, slope 0.93, and intercept +0.345 (S,

yx = 0.170). Linear regression of results by the FETI method (y) and by EMIT allowed the calculation of a correlation coefficient of 0.98, slope 0.954, and intercept −0.05 (S,

yx = 0.148, n = 90). In the comparison between the FETI (y) and the FPIA (x), despite a good correlation coefficient (r = 0.937, n = 62) we observed a consistent bias between the two techniques, the values being lower for FPIA than for FETI (slope 0.802, intercept 0.037, S,

yx = 0.37).

Interfering substances. We evaluated the effect of hemolysis, lipemia, and bilirubin on the FETI method by determining the analytical recovery of digoxin in sera containing high concentrations of these potential interferents. Concentrations of 180 and 300 μmol/L of bilirubin did not significantly affect the recovery of 0.8, 1.6, or 3.2 μg of added digoxin per liter (range of recovery, 94 to 101%). Similarly, the addition of 2 and 6 g of hemoglobin or 4.52 and 9.52 mmol of triglycerides per liter did not interfere with digoxin recovery.

Discussion

Monitoring the concentrations of digoxin in serum is helpful in determining appropriate dosage adjustments, and can be useful in evaluating the individual patient’s response to the drug.

Although RIA methods provide reliable results for measuring digoxin, they are slow and present the disadvantages common to isotopic techniques. On the other hand, the FETI techniques, which are both accurate and precise, are also rapid. The FETI method showed good correlation with both RIA and EMIT methods. Additionally, FETI offers standard curve stability over at least five days and long shelf-life for reconstituted reagents (12 weeks at 2–8 °C, confirming the manufacturer’s claim). The recovery studies demonstrated that the concentration of digoxin measured by FETI is independent of the protein concentration of the sample. Therefore this method is suitable for determining digoxin in sera of patients with abnormal concentrations of serum proteins. By contrast, other immunoassays for digoxin (e.g., FPIA) give accurate results only when the protein concentration of the sample is within the normal range: patients who have a low or high protein concentration or samples that have been diluted with a protein-free diluent may give falsely high results for digoxin (6, 7). This may explain the observed unsatisfactory correlation between the FETI and the FPIA method.

The cross reactivity of the FETI antibody to digoxin with other drugs, endogenous substances, and digoxin metabolites is similar to that of the EMIT antibody; its specificity was previously investigated and was found satisfactory for clinical use (8, 9).

The most common endogenous interferents do not significantly affect the results obtained with this immunoassay. Because it is automated, the FETI method can be used to routinely measure large series of samples in the clinical laboratory. The stability of the standard curve and the reagents permits “stat” determinations of digoxin in emergency situations.

In conclusion, FETI seems to be a reliable technique for monitoring digoxin concentrations in clinical laboratory practice.

References


