Nonisotopic Binding Assay for Measuring Vitamin B₁₂ and Folate in Serum

Jan van der Weide, Hendrika C. Homan, Evelina Cozijnsen-van Rheenen, Yvonne Veldwijk-Kipp, Jan Poortman, and Robert J. Kraaijenhagen

We evaluated a nonisotopic method (CEDIA; cloned enzyme donor immunoassay) for estimating vitamin B₁₂ and folate in serum. The assays were performed with a Cobas Mira analyzer. Intra-assay CVs were from 3.7% to 11.0% for vitamin B₁₂ and from 1.2% to 10.7% for folate. Interassay CVs ranged from 9.5% to 11.9% for vitamin B₁₂ and from 6.1% to 18.5% for folate. Linearity was satisfactory, with analytical recovery of 94% at 8.7 and 25.4 nmol/L for folate and 280 and 554 pmol/L for vitamin B₁₂. The detection limit was 3.6 nmol/L for folate and 12.3 pmol/L for vitamin B₁₂. Results of this assay correlated well with those of a conventional radioassay: r = 0.98 for vitamin B₁₂ (n = 51) and r = 0.97 for folate (n = 57). The CEDIA was easy to perform but appeared to be unreliable for use with samples from myeloma patients.

Additional Keyphrases: CEDIA · radioassay · intermethod comparison · myeloma

Reliable detection of vitamin B₁₂ and folate in serum is important because deficiencies of these nutrients lead to disturbances of normal DNA synthesis and erythrocyte formation (1). Numerous tests, almost all dual-radioisotope assays, have been developed for quantitative detection of vitamin B₁₂ and folate in serum (e.g., 2). We studied the performance of the nonisotopic cloned enzyme donor immunoassay (CEDIA) for determining vitamin B₁₂ and folate concentrations in serum and compared the results with those of a competitive-protein-binding radioassay.

The principle of the CEDIA is as follows: Two fragments of β-galactosidase (EC 3.2.1.23) are prepared by recombinant DNA techniques; when these fragments are mixed, they combine to form the active enzyme (3, 4). One fragment is an enzyme acceptor, and the other fragment is an enzyme donor. The ligand (vitamin B₁₂ or folate) competes with the enzyme donordonor–ligand conjugate for a limiting amount of specific binding protein. The binding of vitamin B₁₂ (by porcine intrinsic factor) or folate (by bovine milk folate-binding protein) will inhibit the reassociation of enzyme donor and acceptor. The concentrations of vitamin B₁₂ and folate in the sample are proportional to the amount of enzyme formed, as monitored by the hydrolysis of chlorophenol-β-D-galactopyranoside at 550 nm in the vitamin B₁₂

We performed the CEDIA with a Cobas Mira analyzer (Roche Diagnostics, Mijdrecht, The Netherlands). The CEDIA vitamin B₁₂/folate kit was purchased from Microgenics (Concord, CA). Immunoassay control solutions, containing 5.9 and 14.0 nmol/L of folate and 300 and 625 pmol/L of vitamin B₁₂ were obtained from Bio-Rad Labs. (Veenendaal, The Netherlands). The radioassay kit used for the dual estimation of vitamin B₁₂/folate was a boiling-type assay from Becton Dickinson (Amersfoort, The Netherlands).

We studied selected patients' samples that were routinely analyzed for vitamin B₁₂ and folate. CEDIA and radioassay reagents were reconstituted according to the suppliers' instructions with one slight modification. Serum samples, standards, and controls were diluted with pretreatment reagent and boiled for 20 min to destroy the binding proteins. After cooling, the boiled samples were centrifuged (1200 × g, 15 min, 4 °C), and the clear supernate was used. The kit instructions recommended centrifuging for 5 min at 3000 × g. To make the CEDIA kit more accessible for smaller hospital laboratories, we decided to examine the feasibility of using a lower centrifugal force and a longer centrifugation time. In our hands, 15 min at 1200 × g was satisfactory. The CEDIA was performed at 37 °C according to the settings recommended by the manufacturer. The radioassay was also performed according to the instructions of the manufacturer. The serum samples were stored at −20 °C until analysis. In a typical CEDIA, three calibrators, containing vitamin B₁₂ at 21, 559, and 1107 pmol/L or folate at 0, 25.2, and 50.5 nmol/L, and as many as 90 patients' samples were analyzed.

Results

Precision. Intra-assay CVs for the vitamin B₁₂ and folate assays are shown in Table 1. Interassay CVs over a two-week period (10 series) are shown in Table 2.

Correlation. Vitamin B₁₂ in 51 samples and folate in

Table 1. Intra-Assay Precision of Vitamin B₁₂ and Folate CEDIA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vitamin B₁₂ (mean (SD), pmol/L)</th>
<th>CV, %</th>
<th>Folate (mean (SD), nmol/L)</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level I</td>
<td>135 (15)</td>
<td>11.0</td>
<td>8.9 (1.0)</td>
<td>10.7</td>
</tr>
<tr>
<td>Level II</td>
<td>555 (58)</td>
<td>10.4</td>
<td>30.6 (1.6)</td>
<td>5.3</td>
</tr>
<tr>
<td>Level III</td>
<td>1050 (38)</td>
<td>3.7</td>
<td>43.9 (0.5)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

n = 10.
Table 2. Interassay Precision of Vitamin B₁₂ and Folate

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vitamin B₁₂ (Mean (SD), pmol/L)</th>
<th>CV, %</th>
<th>Folate (Mean (SD), nmol/L)</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level I</td>
<td>154 (18)</td>
<td>11.9</td>
<td>8.4 (1.6)</td>
<td>18.5</td>
</tr>
<tr>
<td>Level II</td>
<td>513 (56)</td>
<td>10.8</td>
<td>29.5 (3.7)</td>
<td>12.5</td>
</tr>
<tr>
<td>Level III</td>
<td>947 (90)</td>
<td>9.5</td>
<td>42.9 (2.8)</td>
<td>6.1</td>
</tr>
</tbody>
</table>

n = 10 series, over two weeks.

57 samples were assayed by the radioassay and the CEDIA. Correlation between the two methods was made by the least-squares method. For vitamin B₁₂ the correlation coefficient was 0.98, the slope was 0.90, the intercept 55.0 pmol/L, and S\(^{XY}\) was 30 pmol/L. For folate the correlation coefficient was 0.97, the slope was 1.45, the intercept was 1.8 nmol/L, and S\(^{XY}\) was 2.2 nmol/L.

Recovery. Analytical recovery was assessed by adding CEDIA standards in various concentrations to serum samples containing small concentrations of vitamin B₁₂ and folate. We then measured the vitamin B₁₂ and folate concentrations with the CEDIA. Analytical recovery was calculated as [(amount after addition − amount before addition)/amount added] × 100%. Recovery of added vitamin B₁₂ standards of 280 and 554 pmol/L was 94%. For added folate standards of 8.7 and 25.4 nmol/L, recovery was also 94% (triplicate measurements).

Calibration. The standards used in the radioassay were also measured in the CEDIA. Folate values were almost identical at all the concentrations investigated. However, vitamin B₁₂ values were always considerably higher: 0 vs 310, 148 vs 384, 295 vs 521, and 738 vs 990 pmol/L by radioassay and CEDIA, respectively, for a typical experiment.

Linearity. The linearity of the CEDIA for vitamin B₁₂ and folate was determined with a serial dilution of a serum pool with zero diluent (Dupont, 's Hertogenbosch, The Netherlands) for folate and with serum from a vitamin B₁₂-deficient patient for vitamin B₁₂. Five separate dilutions were assayed, and each dilution was run in duplicate. The response varied linearly with concentration up to the highest-concentration standard available, i.e., 1107 pmol/L for vitamin B₁₂ and 50.8 nmol/L for folate.

Sensitivity. We assessed analytical sensitivity by 10 replicate measurements of the zero diluent for folate and of a 15.3 pmol/L standard of vitamin B₁₂ in a single run. The minimal detectable concentration, defined as the mean + 3 SDs, was 3.6 nmol/L for folate and 12.3 pmol/L for vitamin B₁₂.

Interference of paraproteins. The CEDIA for vitamin B₁₂ appeared to be unreliable for serum samples from myeloma patients. In about half of the samples (4/7), we found a marked discrepancy between the radioassay and the CEDIA (Table 3).

Discussion

The principle of the CEDIA is well established. It has already been applied for determining digoxin (4, 5), vitamins, hormones, and tumor markers (6).

Table 3. Assay Comparison for Vitamin B₁₂ (pmol/L) in Serum from Seven Myeloma Patients

<table>
<thead>
<tr>
<th>Type of multiple myeloma</th>
<th>Radioassay</th>
<th>CEDIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG κ</td>
<td>40</td>
<td>692</td>
</tr>
<tr>
<td>IgG κ</td>
<td>200</td>
<td>885</td>
</tr>
<tr>
<td>IgG κ</td>
<td>165</td>
<td>45</td>
</tr>
<tr>
<td>IgG λ</td>
<td>210</td>
<td>214</td>
</tr>
<tr>
<td>IgA κ</td>
<td>290</td>
<td>996</td>
</tr>
<tr>
<td>IgA λ</td>
<td>185</td>
<td>220</td>
</tr>
<tr>
<td>Bence Jones</td>
<td>355</td>
<td>316</td>
</tr>
</tbody>
</table>

We investigated the CEDIA for vitamin B₁₂ and folate and compared our results with the conventional radioassay. The results from the nonisotopic assay correlated closely with those from the radioassay for both vitamin B₁₂ and folate. The correlation was excellent over the clinically relevant range. With the Cobas-Mira, the CEDIA method was easy to handle. Series of as many as 100 tests could be analyzed in one run because of the kit size. With a detection limit of 12.3 pmol/L for vitamin B₁₂, the CEDIA surpassed the radioassay in sensitivity. Intra-assay and interassay CV studies showed that the CEDIA is comparable with the currently available methods. The intercepts and slopes for the correlations of the methods may be caused by differences in standardization, standards, or (most likely) matrix and binding characteristics. Fu and Hesbemi (7) recently reported the same phenomenon. When radioassay standards of vitamin B₁₂ were measured in the nonisotopic assay, the measured amounts were always greater than the expected values. It is tempting to speculate that vitamin B₁₂ is released more easily from a synthetic matrix (in standards) than from the endogenous matrix (in serum).

The only drawback of the CEDIA system is its failure to reliably measure vitamin B₁₂ in serum from myeloma patients. We came across this phenomenon by chance. In our series of patients' samples for the correlation study, there was one sample from a myeloma patient (IgG κ). We found a large difference between the two methods for this sample. In a preliminary experiment we selected seven different myeloma samples, as indicated in Table 3. In about half of the samples we found a marked discrepancy between the assays: values from the CEDIA were higher (as much as 17-fold) and lower (fourfold) than the measured values in the radioassay.

From hematological indexes and the peripheral blood smear, we concluded that these patients should have had normal values for vitamin B₁₂, and therefore it is likely that the CEDIA failed in these cases. As mentioned in the kit instructions, gamma globulins added to serum with a normal albumin concentration do not disturb the assay up to a total protein concentration of 100 g/L. In severe myeloma, the protein concentration is sometimes much higher. It also is possible that M components have an affinity for substances in the assay, which may contribute to the problems we found. Because protein concentrations can be decreased and increased in myeloma samples, we conclude that the CEDIA is not suitable for determining vitamin B₁₂ in these samples.
The price of a CEDIA determination of vitamin $B_{12}$ and folic acid is $\sim $8.50 per sample, whereas the radioassay costs $\sim $3.50. Advantages of the CEDIA over the radioassay are that laboratory personnel are not exposed to ionizing radiation from the radionuclides used, duplicates of samples can be run simultaneously, the analysis is much faster (twofold), and the technique is applicable for routine use in laboratories without a radioactivity license.

References

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Design and Evaluation of an Anti-Evaporative Cover for Use with Liquid Containers

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We have designed an anti-evaporative cover for use with the sample and reagent cups and other liquid containers that are required in automated analytical systems. This cover, which is simple in design, consists of a baseplate and a cylindrical chimney. By increasing the height and decreasing the inner diameter of the chimney, evaporative losses can be reduced to $<$0.1%/h; thus, aliquots of sample and reagents can be allowed to remain in their cups and containers for several hours before an analytical error due to evaporation will be measurable. We have also modified a model that we previously developed and validated to estimate evaporative losses from open sample cups, to allow us to predict evaporative losses from cups and containers fitted with the new type of cover. This model confirms that the magnitude of the evaporative loss is inversely proportional to the resistance provided by the headspace above the sample in the cup and by the space defined by the chimney of the cover. With chimney heights ranging from 12 to 36 mm and their inner diameters from 1 to 4 mm, >70% of the resistance to evaporation is provided by the cover.

Additional Keyphrases: sample handling variation, source of error

Previous studies have demonstrated that the evaporative loss of volatile components from a sample in a sample cup can have a significant effect on analytical accuracy, because the concentrations of nonvolatile analytes increase (1–5) and the concentrations of the volatile analytes decrease (7, 8) in the sample. Depending on environmental conditions such as temperature, humidity, ambient air flow, and the geometry of the cup, analytical errors of 1–10% have been reported (6).

Analytical error has always been a concern to laboratorians and manufacturers of diagnostic equipment (9–11); however, it is now of particular importance, given the implementation of new laboratory regulations (12) in the Clinical Laboratory Improvement Act (CLIA) of 1988. As mandated by these regulations, each clinical laboratory must demonstrate acceptable performance; failure to do so can result in loss of accreditation and revocation of its legal license to operate. Consequently, the new regulations will require laboratorians and manufacturers of diagnostic equipment to reassess their analytical goals (13, 14) and take proactive steps to reduce all sources of analytical error.

Evaporative loss is one source of analytical error that must be minimized in light of the new CLIA-88 regulations. For example, to achieve a passing grade for a proficiency testing (PT) challenge for serum sodium, a laboratory must report a measured value that falls within a range defined by the target value $\pm 4$ mmol/L (12). Thus, if a PT sample has a sodium concentration of 140 mmol/L, an evaporative loss of only 3% will lead to overestimation of the sodium concentration and failure of the PT challenge.

Various measures have been used to minimize the effect of sample evaporation on analytical error. These include minimizing the residence time of the sample in its cup (15, 16); selecting a sample cup with optimal geometry (5); maintaining an optimal liquid level within the cup (4, 5); and protecting the surface of the sample. Techniques used in this latter category include layering the sample surface with silicone oil (17, 18), protecting the sample surfaces by placing a cover over the entire sample carousel or tray (3, 19), or capping the

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