Chemiluminescence Receptor Assay for Measuring Vitamin B\textsubscript{12} in Serum Evaluated

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We evaluated a chemiluminescence receptor assay for vitamin B\textsubscript{12} in serum (Magic Lite; Ciba Corning Diagnostics), in which an acridinium ester label is used with magnetic particle separation. Within- and between-batch precisions were generally acceptable, except at low analyte concentrations. The reference range determined from 104 elective preoperative patients was 120–610 pmol/L, compared with 150–590 pmol/L for our in-house radioligand-binding assay. Magic Lite discriminated between normal and abnormal results as effectively as the in-house method when local reference ranges were applied. Magic Lite demonstrated a negative bias at low analyte concentrations and was unable to detect any vitamin B\textsubscript{12} in two deficient patients. Assay accuracy—judged from analyte recovery and comparisons with the in-house method and two other radioassay kits (Quantaphase, Bio-Rad Labs., and Immophase, Ciba Corning Diagnostics)—was poor at low B\textsubscript{12} concentrations when the manufacturer’s recommended two-point calibration was used. This problem was partially corrected by using a full set of calibrators.

**Indexing Terms:** intermethod comparison/radioligand-binding assay compared/nutritional status

Since its introduction in 1960, radioassay (1) has been a valuable diagnostic tool because of its excellent sensitivity, specificity, and wide applicability to biological analytes. However, given the limitations of using radioactive tracers—short reagent life, licensing requirements, user safety, and disposal of radioactive compounds—nonisotopic alternatives have been sought (2). One such alternative has been to replace the radioisotopic tracer with a chemiluminescent acridinium ester.

We have evaluated a vitamin B\textsubscript{12} assay (Magic Lite\textsuperscript{5}; Ciba Corning Diagnostics, Medfield, MA), in which an acridinium-ester-coupled cyanocobalamin molecule is used as the tracer in a competitive receptor assay. We first investigated each step of the procedure and then performed a clinical and analytical assessment by comparison with an in-house radioligand binding assay (RLBA) that has been in use in our laboratory for the past 10 years.

Because no definitive method for vitamin B\textsubscript{12} is available to assess method accuracy, we investigated accuracy further by using two other RLBA commercial kits.

One was Immophase (Ciba Corning), the radioreceptor assay chosen by Ciba Corning for the evaluation of their Magic Lite system. The other, Quantaphase (Bio-Rad Labs., Richmond, CA), is the vitamin B\textsubscript{12} kit currently used by 75% of the laboratories in Ontario that participate in the Ontario Medical Association Laboratory Proficiency Testing Program.

**Materials and Methods**

In-House B\textsubscript{12} Radioassay Method

The in-house method for vitamin B\textsubscript{12} is based on the technique described by Raven et al. (3), modified to include a cobanamide block added in excess to the crude intrinsic factor binder and with the assay pH changed to 9.3 (4). Serum is boiled in an acid cyanide buffer (pH 4.6), cooled, and centrifuged. Borate buffer is added to an aliquot of serum extract to raise the pH to 9.3. Radiolabeled vitamin B\textsubscript{12} is added, followed by blocked hog intrinsic factor. After a 90-min incubation at room temperature, an albumin-coated charcoal suspension is added to separate the bound from the free ligand and the samples are incubated at room temperature for 15 min. The samples are then centrifuged, the supernates are decanted, and their radioactivity is counted on a gamma counter. Results are determined from an eight-point standard curve (range, 20–1400 pmol/L) prepared in a bovine serum albumin base, and assayed along with the patients’ specimens. A four-parameter log-logit data reduction system is used to calculate results. The assay calibration curve is linear over the range 20–1400 pmol/L. The detection limit (mean + 2 SD for the zero standard) is 20 pmol/L. The assay reference range, determined 10 years ago as the 2.5 and 97.5 percentiles from 147 healthy subjects, is 135–500 pmol/L.

**Commercial Kits**

Magic Lite is a competitive chemiluminescence receptor assay. All steps are performed at room temperature. The serum sample is pretreated with sodium hydroxide for 15 min to release all the B\textsubscript{12} from endogenous binding proteins. Sample and solid phase (purified hog intrinsic factor coupled to paramagnetic particles) are incubated together for 45 min and then, after the addition of acridinium ester-labeled B\textsubscript{12}, for another 30 min. Bound and unbound B\textsubscript{12} are separated by placing the tubes in a magnetic separator for 3 min and then decanting the supernate. The pellet is washed and resuspended before its chemiluminescence is measured in the Magic Lite Analyzer. Oxidation of the acridinium ester by addition of alkaline hydrogen peroxide, automatically performed in the analyzer, initiates chemiluminescence. The light emission is expressed as photon counts (relative light units, RLU) accumulated.

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during a 2-s counting time. Two calibrators, one low and one high, are used as recommended by the manufacturer in each assay. Their RLU values are used along with a stored calibration curve defined for each lot of reagents. A supplementary set of six additional calibrators is available from the manufacturer and can be used in place of the two-point calibration method.

For the full calibration curve, the analyzer uses a spline function to fit a logit transformation of RLU against log concentration.

Immophase and Quantaphase, both RLBAs, are performed by combining a serum sample, vitamin B₁₂ (⁵⁷Co-labeled), and a solution containing dithiothreitol and cyanide at pH 9.2. The mixture is boiled, cooled, and then combined with immobilized purified hog intrinsic factor. After a 1-h incubation at room temperature, the samples are centrifuged and decanted, and the radioactivity of the pellets is counted with a gamma counter. Results are determined from a six-point calibration curve prepared with a human serum albumin-based calibrator and assayed with the patients' specimens. A four-parameter log-logit data reduction system is used to calculate results. Both kits determine B₁₂ and folate simultaneously, but for the purposes of this study we have considered only the B₁₂ assay.

All assays were performed as recommended by the manufacturer. All samples, calibrators, and controls were assayed in duplicate.

Control Materials

We used Ciba Corning Tri-Level Ligand control levels A, B, and C, which are recommended by the manufacturer for use with the Magic Lite kit, and RIATRAC Plus Level 1 from Becton Dickinson Advanced Diagnostics (Sparks, MD). Quality-control material was stored and used according to the manufacturers' instructions. In addition, we used four serum pools with B₁₂ values of −50, 110, 250, and 540 pmol/L, prepared for use as quality controls in the in-house method. These pools were aliquoted, frozen at −20°C, and thawed once just before use.

Reference Interval and Clinical Samples

Reference interval samples were collected from 104 elective preoperative adult patients (44 men, median age 60, range 19–75; 60 women, median age 30, range 18–84) and assayed to determine the reference intervals for the Magic Lite assay. Although the reference interval had been previously established for the in-house assay, it was reassessed with samples from this population.

A patient comparison group was selected from specimens submitted to our laboratory for routine B₁₂ assay. The group comprised 50 samples selected to have approximately evenly spaced values over the range 20–1200 pmol/L, as measured by the in-house method. These samples were assayed in at least five separate runs over a 3-week period. All serum samples were frozen in aliquots until assay.

Performance Studies

Precision. Within-batch precision was assessed for Magic Lite and the in-house assay for two sets of samples: (a) replicate analysis (10 determinations) of each of three human sera and (b) duplicate analysis of the 104 reference population samples and the 50 clinical samples.

Between-batch precision for Magic Lite was obtained by assaying the four serum pools and the RIATRAC Plus Level 1 in eight runs. Between-batch precision data for the four pools and RIATRAC were already available on a monthly basis for the in-house method.

Recovery. Analytical recovery was assessed after adding to aliquots of a low-B₁₂ serum sample various amounts of a cyanocobalamin solution (crystalline cyanocobalamin; Sigma Chemical Co., St. Louis, MO) diluted in buffered human serum albumin, and assaying the sera before and after the additions.

Linearity. Three patients' samples were assayed at dilutions corresponding to 20–100%. Magic Lite B₁₂ fluluent, available from Ciba Corning as a "buffer containing human serum albumin," and recommended for use with Magic Lite as a diluting agent, was used to prepare dilutions. Data were corrected for the endogenous concentrations of B₁₂ in the diluent as directed in the manufacturer's package insert.

Within-assay drift. Ciba Corning Tri-Level Ligand controls A, B, and C were assayed at the beginning and at the end of five consecutive runs that contained as many as 120 tubes each.

Accuracy comparisons between kits. Six serum samples were assayed in replicate by all four assay methods. The validity of two-point calibration for Magic Lite was assessed by running a supplementary set of six calibrators supplied by Ciba Corning. The manufacturer quotes values of "<60," 37 ± 33, 74 ± 39, 185 ± 49, 553 ± 94, and ">1057 pmol/L." The low and high "standards" as supplied are unusable as calibrators because no concentration can be attributed to them. For the other four points, the quoted mean value was treated as the assay value. On the basis of telephone instructions from the manufacturer (personal communication), we assigned values of 0 and 1496 pmol/L to the "<60" and ">1057" pmol/L standards, respectively. The Magic Lite run was then recalibrated by using the two supplied calibrators, the six supplementary calibrators, and the full standardization protocol option on the Magic Lite Analyzer.

Results

Time Dependence of Magic Lite Assay Steps

The effects of varying the duration of the incubation times for the releasing, the solid phase, and the acridinium ester-labeled B₁₂ steps were evaluated by assaying three patients' sera having low, normal, and high concentrations of vitamin B₁₂.

At the recommended time of 15 min, the release of vitamin B₁₂ was maximum. The solid-phase ligand binding to intrinsic factor had reached ~70% completion at the recommended time of 45 min. The acridinium
ester-labeled B₁₂ ligand binding to intrinsic factor was ~60% complete at the recommended time of 30 min (Fig. 1). The failure of the latter reaction to reach completion is especially noticeable for the sample giving high RLU values, i.e., that with a low concentration of the vitamin, for which the reaction had still not reached completion at 120 min.

We assessed the magnetic separation step before washing by measuring the vitamin B₁₂ level in each of three sera, using a separation time of 30 s, 3 min (recommended by the manufacturer), and 10 min. After 30 s, the aggregation of magnetic particles was incomplete and all results were too high. Increasing the separation step from 3 to 10 min did not yield any significant change in results.

Separating, washing, and resuspension were all assessed by comparing a 5-s vortex-mixing to the three 5-s vortex-mixings recommended by the manufacturer. We found that results for a single 5-s vortex-mixing were indistinguishable from those for the recommended protocol.

Determination of Reference Interval

The 104 reference samples had a unimodal and positively skewed distribution. The fifth and 95th percentiles were 150 and 590 pmol/L for the in-house method and 120 and 610 pmol/L for Magic Lite, respectively. The Magic Lite manufacturer's reference interval was 177–812 pmol/L. Sixteen of our reference samples had results of 120–176 pmol/L, which would have been low by the manufacturer's criteria but not by our locally derived criteria.

The patient comparison group was classified with the reference limits appropriate to each method. Fifteen samples were low by the in-house method and by Magic Lite, according to the locally derived reference interval. Two other samples would have been classified as low by the manufacturer's lower reference limit. Two samples in the low group had results below the zero point on the calibration curve for the Magic Lite assay, compared with 20 and 40 pmol/L by the in-house method.

Linear regression analysis of the combined data from the 50 patient comparisons and the 104 reference interval samples for Magic Lite vs in-house gave a correlation coefficient of 0.98, an intercept of -6.37 pmol/L (SE 8.42, P < 0.001), and a slope of 1.13 (SE 0.019, P < 0.001).

Precision

Within-batch precision (CV) derived from replicate assays (n = 5) of three sera with mean values of 96, 364, and 619 pmol/L was 4.9%, 4.0%, and 2.0%, respectively, for the in-house method, and 19.7%, 3.4%, and 2.0%, respectively, for the Magic Lite method.

Similar precision data were derived from the results of duplicate assays of 126 samples having mean values >150 pmol/L; CVs were 1.8% for the in-house assay and 3.5% for the Magic Lite method. Fig. 2 illustrates the difference between duplicates, expressed as a percentage of their mean, for the in-house method and for Magic Lite. Low values were excluded from this Figure because the low bias of the Magic Lite method accentuated duplicate differences when expressed as a percentage of mean value. Poor precision by Magic Lite was also seen at normal analyte concentrations, with 14 samples having a difference between duplicates of >10% of the mean, compared with 2 samples for the in-house method.

Between-batch precision for the pools with borderline, normal, and high values (pools A, B, and C, respectively) was comparable for both methods (Table 1). Magic Lite, however, had poor precision at low concentrations of vitamin B₁₂.

Other Studies

Analytical recovery. By the Magic Lite method, the recovery of 100, 369, and 748 pmol/L added to a base pool containing 66 pmol/L vitamin B₁₂ was 75%, 102%, and 116%, respectively.

Linearity. In all cases, the relationship between concentration and dilution of sera did not significantly deviate from linearity over the range of concentrations studied (100 to 1100 pmol/L).

Within-run drift. No significant within-run drift was
Table 1. Between-batch precision of results with control materials.

<table>
<thead>
<tr>
<th>In-house (n = 27)</th>
<th>Magic Lite (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) B₁₂, pmol/L</td>
<td>Mean (SD) B₁₂, pmol/L</td>
</tr>
<tr>
<td></td>
<td>CV, %</td>
</tr>
<tr>
<td>Low pool</td>
<td>52 (6.1)</td>
</tr>
<tr>
<td>RIA TRAC</td>
<td>107 (10.7)</td>
</tr>
<tr>
<td>Pool A</td>
<td>111 (7.7)</td>
</tr>
<tr>
<td>Pool B</td>
<td>250 (13.7)</td>
</tr>
<tr>
<td>Pool C</td>
<td>540 (25.0)</td>
</tr>
</tbody>
</table>

* Detection limit (0 standard + 2SEM), mean of nine replicates: 9 pmol/L.

observed. In all cases, differences were within the anticipated within-run precision envelope.

Accuracy comparisons between kits. Results of the patient comparisons made with the four methods and with the two-point and the eight-point calibrations for Magic Lite are presented in Table 2.

Discussion

The upper reference interval limits were similar for both methods, but the lower limits were lower for Magic Lite. The Magic Lite locally derived reference interval of 120–620 pmol/L differs considerably from that provided by the manufacturer (177–812 pmol/L), which is stated to be based on results for 262 normal subjects. Twenty-one percent of our apparently healthy reference population would have fallen into the B₁₂-deficient group, had we used the manufacturer’s reference limits. This emphasizes the importance of each laboratory deriving its own reference range.

The ability of Magic Lite to discriminate between low, borderline, normal, and high results was similar to that of the in-house method when the local reference intervals were applied. The magnitude of the low values was considerably less by Magic Lite, with two values falling below zero on the calibration curve. For values >800 pmol/L, Magic Lite gave slightly higher results than the in-house method.

Both methods were comparable in discriminating between normal and abnormal results. The in-house method included a cobainamide block, methodology known to have a slightly higher lower limit of normal than RLBA methods that use purified intrinsic factor (5).

Magic Lite within-batch precision was generally acceptable, but low values and occasional normal values duplicated poorly. Neither the solid phase nor the acridinium ester-labeled B₁₂ binding steps reached equilibrium in the incubation time recommended in the kit procedure; this, together with the multiple steps of the assay, may have contributed to the poor precision. The manufacturer emphasizes that a consistent technique is very important to achieve reproducible results. But even when the utmost care was taken by a single skilled operator—using a consistent pipetting technique; avoiding foaming or bubbles; and using a slow, smooth decanting action—spurious results could not be avoided. The Tri-Level ligand controls recommended by the manufacturer showed acceptable precision but were of no use in assessing precision at low concentrations of vitamin B₁₂ because the lowest of these had a mean of 186 pmol/L, well above the lower reference limit of 120 pmol/L.

The 75% recovery of the added 100 pmol/L vitamin B₁₂ solution reflects the apparent underestimation of vitamin B₁₂ at low concentrations. Linearity was satisfactory when the manufacturer’s diluent was used to ensure absence of compounding kit-specific matrix effects.

Apparently the imposed shape of the standard curve based on two-point calibration is responsible for the discrepancy between Magic Lite and other methods. When a full eight-point curve is used (Table 2), the discrepancy at low concentrations disappears.

We conclude that the Magic Lite chemiluminescence assay is an adequately sensitive assay that overcomes some of the disadvantages of isotopic methods. However, either the multiple steps of the assay or some attribute of its chemistry appears to make it prone to operator-induced variables. A two-point calibration is inadequate to ensure accuracy at low levels. A fully automated version of this kit has been developed that may overcome some of these hazards. Finally, if the provided Tri-Level ligands are used for quality control, it is essential to add an additional control, with a low B₁₂ value.

We thank Ciba Corning Diagnostics for lending us the instrumentation and donating the reagent kits, standard sets, B₁₂ diluent, and Tri-Level Ligand controls.

References

Table 2. Methods comparison.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Magic Lite</th>
<th>Mean B₁₂ conc, pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Quanti-phase</td>
</tr>
<tr>
<td>1</td>
<td>77</td>
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</tr>
<tr>
<td>2</td>
<td>107</td>
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<td>5</td>
<td>834</td>
<td>931</td>
</tr>
<tr>
<td>6</td>
<td>950</td>
<td>1027</td>
</tr>
</tbody>
</table>

n = 6. All others, n = 9 each.

⁰ Significantly different from in-house result (P < 0.05).