Microbiological Assay for Vitamin B₁₂ with Use of a Colistin-Sulfate-Resistant Organism

Brian P. Kelleher,¹ Kieran G. Walshe,² John M. Scott,³ and Sean D. O'Brien¹ ³

In this simplified microbiological assay for serum vitamin B₁₂, Lactobacillus leichmanii (NCIB 8117) adapted to tolerate high concentrations (500 mg/L) of the polymyxin antibiotic colistin sulfate is used. Results were similar in parallel experiments in which we used both the parent strain of L. leichmanii (NCIB 8117), and the new adapted strain. Evaluation of assay performance showed excellent analytical recovery of added cyanocobalamin (97%, SD 3%) and good interassay and intra-assay precision (CV <5%). This modified assay system obviates the need to sterilize culture medium and glassware. Consequently, assay manipulations may be carried out openly, without aseptic precautions. Moreover, this adapted organism would be suitable for use in an automated microbiological assay system.

Microbiological assays of vitamin B₁₂ in serum have been extensively used as an index of cobalamin status in man and have consistent results, with good clinical correlations (1–4). The stringent aseptic precautions necessary in such assays (5, 6) make them both long and tedious. The development of radiolabile dilution assays for vitamin B₁₂ in the 1980s (7, 8) led to the widespread use of several different commercially available kit systems. However, radiolabelling assays based on the use of intrinsic factor as a competitive cobalamin binder have given clinically misleading results (9, 10) and continue to do so despite considerable modifications of methodology (6).

The development of an assay for folates in which a chloramphenicol-resistant strain of Lactobacillus casei was used greatly simplified that assay (11). The objective of the present study was to develop a similar type of assay for vitamin B₁₂. Such an assay would be both clinically reliable and easily performed in the general diagnostic laboratory.

Materials and Methods

Materials

Colistin sulfate (USP grade) was obtained as a sterile powder from Pharmax Limited, Bexley, Kent, U.K. Vitamin B₁₂ medium (USP grade) was obtained from Difco Laboratories, Detroit, MI 48220. Lactobacillus leichmanii (NCIB 8117) was obtained from Turr Research Station, Aberdeen, Scotland. All other chemicals used were of reagent grade. Glass-distilled water was used throughout. Assays were completed in 13 × 100 mm glass culture tubes, and we made additions of extract with a “Steeper” automatic pipette (Socorex, 1020, Renens, Switzerland). Tubidimetric readings were made in a Beckman Model 35 spectrophotometer (Beckman, High Wycombe, Bucks HP124 SL, U.K.).

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10 min) and resuspension in 10 mL of sterile water. Inoculum strength was standardized turbidimetrically. After 50-fold re-dilution of inoculum with sterile water, the appropriate assay tubes were inoculated dropwise and incubated at 37 °C for 42 h.

A comparison of two assays. We compared the results for serum vitamin B₁₂ by both the colistin-sulfate-based assay as described and a conventional method in routine use in our laboratory. The conventional method was based on that of Spray (2), modified to include a final autoclaving (115 °C, 6 min) of the tubes containing medium and vitamin B₁₂ extracts before inoculation.

We determined the consistency and reproducibility of values for control serum and the analytical recovery of added cyanocobalamin with this assay procedure.

Results

Figure 1 shows response curves of the organism to cyanocobalamin in media containing various concentrations of colistin sulfate. A colistin sulfate concentration of 100 mg/L was chosen for use in the routine assays because the concentration drop from 500 mg/L to 100 mg/L gives a good growth response while maintaining sterility.

We evaluated, in parallel assays, the comparative responses of both the colistin-sulfate-resistant organism and the parent strain, using aliquots of the same extracts, medium, and standards for both (Figure 2). The coefficient of correlation (r) was 0.97 (n = 84), which is highly significant (p <0.001). A subsequent comparison of 90 serum vitamin B₁₂ values estimated by both the present assay and the conventional technique gave similar results (Figure 3): r was 0.98 (p <0.001).

The performance data for interassay and intra-assay variations are shown in Table 1. Precision between and within assays was good, CVs being <5% in both. Analytical-recovery experiments were performed by adding known amounts of cyanocobalamin standard to 10 samples at each

Discussion

Megaloblastic anemia in man as a result of deficiency in either folate or vitamin B₁₂ presents with identical hematological findings, but it is essential to distinguish between these deficiencies because low cobalamin status may cause irreversible neurological damage. In addition, inappropriate treatment of vitamin B₁₂ deficiency with folate can mask or exacerbate the nerve damage associated with such a deficiency. Cobalamin concentrations in serum, when measured by microbiological assay, correlate well with clinical find-
Table 1. Reproducibility of Control Serum Values as Estimated by the New Method

<table>
<thead>
<tr>
<th>Mean ng/L</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>4.67</td>
<td>3.38</td>
</tr>
<tr>
<td>264</td>
<td>8.64</td>
<td>3.27</td>
</tr>
<tr>
<td>355</td>
<td>12.13</td>
<td>3.42</td>
</tr>
<tr>
<td>495</td>
<td>23.64</td>
<td>4.78</td>
</tr>
<tr>
<td>Interassay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>3.80</td>
<td>3.06</td>
</tr>
<tr>
<td>224</td>
<td>9.01</td>
<td>4.02</td>
</tr>
<tr>
<td>370</td>
<td>14.30</td>
<td>3.86</td>
</tr>
<tr>
<td>504</td>
<td>24.39</td>
<td>4.84</td>
</tr>
</tbody>
</table>

n = 10, throughout.

Table 2. Analytical Recovery of Added Cyanocobalamin from Serum

<table>
<thead>
<tr>
<th>Added ng/L</th>
<th>Measured</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>95</td>
<td>95.00</td>
</tr>
<tr>
<td>200</td>
<td>192</td>
<td>96.00</td>
</tr>
<tr>
<td>400</td>
<td>407</td>
<td>101.75</td>
</tr>
<tr>
<td>800</td>
<td>767</td>
<td>95.88</td>
</tr>
</tbody>
</table>

n = 10 throughout.

ings (3, 4). Nowadays, however, most diagnostic laboratories estimate serum vitamin B₁₂ by radiodilution assay. Assay kits are commercially available and easy to use, whereas the technical difficulties associated with microbiological assays have confined their use to large hospitals and teaching establishments (5, 6). The observation that radiodilution assays in which impure intrinsic factor is used as a binder will lead to a misdiagnosis of pernicious anemia in patients (9, 10) has led to significant modifications of assay systems (12, 13). Despite these modifications, misdiagnoses still occur (6, 14). This has led to an erosion of confidence in the reliance that can be placed on serum vitamin B₁₂ estimations and has prompted unnecessary additional diagnostic testing (6).

The performance of the colistin-sulfate-resistant *L. leichmannii* compared well with that of the parent strain (NCIB 8117) in parallel assays. The assays were similar—the same serum extracts and standards are used—but colistin sulfate at a concentration of 100 mg per liter was included in media dispensed for the resistant organism. Correlation of 84 results for serum vitamin B₁₂ was good (Figure 2), as was analytical recovery of added cyanocobalamin (97%, SD 3%) and precision (Tables 1 and 2).

The present microbiological assay for serum vitamin B₁₂ offers considerable advantages over traditional microbiological assay techniques. *L. leichmannii* (NCIB 8117) has an inherent resistance to the antibiotic and may be easily adapted to tolerate high concentrations (Figure 1). Aseptic precautions are not needed. Medium need not be sterilized by autoclaving and is thus inoculated in bulk before dispensing, rather than by addition of inoculum to individual tubes. Such bulk "seeding" of medium is not only more convenient, it also produces less tube-to-tube variation. Tubes may now be "capped" by covering whole racks with "Parafilm," obviating the use of individual caps that must be recycled. Over 8000 routine estimations of vitamin B₁₂ in serum have been completed by use of this simplified microbiological assay; in no instance has any extraneous bacterial contamination been observed.

This modified microbiological assay is ideally suited for use in the general diagnostic laboratory. It offers simplicity of assay technique and the clinical accuracy associated with more conventional microbiological assays. The colistin-sulfate-resistant organism would also be suitable for use in automated microbiological assay systems.

References