BRIEF REPORT

Rapid Determination of Vitamin B_{12} Binding \( \alpha \) and \( \beta \) Globulin in Serum

By Ellen Silverstein* and Victor Herbert

Study of vitamin B_{12}-binding globulins and unsaturated B_{12}-binding capacity (UBBC) has revealed them to be quantitatively abnormal in a number of conditions.¹⁻⁵ Normal serum contains 200–900 picograms of B_{12} per ml. and has two vitamin B_{12}-binding proteins, an \( \alpha \)- and a \( \beta \)-globulin. The UBBC, expressed as picograms of B_{12}, normally ranges from 1000 to 2200 picograms/ml. Determination of the UBBC may be made rapidly by the method of Gottlieb et al.,⁶ using Co^{60}B_{12} and coated charcoal.

Determination of the UBBC and the ratio of the B_{12}-binding \( \alpha \) and \( \beta \)-globulins has been a useful diagnostic tool in helping to differentiate myeloproliferative disorders from leukocytosis,⁴⁻⁵ polycythemia vera from secondary polycythemia⁷ and in following the course of disease during therapy.⁷⁻⁸ Therefore, a simple method to determine the value of B_{12}-binding \( \alpha \) and \( \beta \)-globulins was sought to replace the more cumbersome column chromatography and electrophoretic technics that have been used in the past to separate B_{12}-binders.⁵⁻⁸ Such a method is described in this report: rapid batch separation using DEAE cellulose.

Materials and Methods

Specimens

Serum and/or heparinized plasma from normal individuals and patients with chronic myelogenous leukemia (CML) and polycythemia vera (PV) was frozen after collection and stored at -20 C.

Preparation of Serum for Batch Separation

A working solution of 10 ng. radioactive Co^{57}B_{12}/ml. in distilled water was prepared from the stock Co^{57}B_{12} (Co^{57}B_{12}, with specific activity 10 mc./mg., E.R. Squibb & Sons)

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*Work performed during the tenure at The Mount Sinai Hospital and School of Medicine of a Summer Research Traineeship of the Research Foundation of the State of New York, as a medical student at the State University of New York—Downstate Medical Center.

Supported in part by United States Public Health Service Research Grants AM 09564 and AM 09062; by the List, Machlin, and Lowenberg Funds, and by City of New York Health Research Council Career Scientist Award 1-435 to V. H.

First submitted Sept. 7, 1967; accepted for publication January 16, 1968.

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Blood, Vol. 31, No. 4 (April), 1968
and kept in a cool, dark place. Equal amounts, 0.25 ml. (2.5 ng.) of the working solution of Co\textsuperscript{57}B\textsubscript{12} were added to 0.25 ml. of each sample of serum or heparinized plasma to be separated. (Such amounts of Co\textsuperscript{57}B\textsubscript{12} were in excess of the UBBC, as determined by the method of Gottlieb et al.\textsuperscript{6} when the UBBC was less than 9.9 ng./ml.). The mixture of Co\textsuperscript{57}B\textsubscript{12} and serum (or plasma) was incubated at 37 C. for 30 minutes and then mixed briefly with a 25 mg. hemoglobin-coated charcoal pellet\textsuperscript{8} to remove free radioactive B\textsubscript{12}. The charcoal was removed by centrifugation and radioactivity in the supernate determined in a well-type scintillation detector. Distilled water, 0.25 ml., was then added to the supernates so that the serum (or plasma) was diluted to one-third its initial ionic strength before application to DEAE.

**DEAE Batch Separation Procedure**

The separation procedure was carried out in new, clean 16 × 100 mm. test tubes using DEAE cellulose (Selectacel ion exchange cellulose, #70, DEAE Standard, Carl Schleicher & Schull Co., Keene, New Hampshire). The DEAE cellulose was used either as a dry, untreated powder, or as a pellet, made from a one-third dilution of a stock slurry of washed DEAE. The stock slurry was prepared as described by Retief et al.\textsuperscript{8} with washes of 1 N NaOH, distilled water and 0.02M phosphate buffer, pH 6.35. For the batch separation, a 1 ml. pellet of washed DEAE in 0.02M phosphate buffer, pH 6.35 was used (about 10 ml. of the one-third dilution of stock DEAE in 0.02M phosphate buffer was centrifuged to make the pellet).

The alternate procedure, producing equally good separations of B\textsubscript{12}-binding globulins, employed 0.170 gms. of dry, untreated DEAE, weighed into a 16 × 100 mm. test tube and prewetted by simply adding 2.0 ml. of 0.02M phosphate buffer, pH 6.35.

The samples of serum, prepared as stated above, were added to the DEAE pellet. The DEAE and serum were mixed by brief gentle agitation of the test tube and allowed to stand for 10 minutes at room temperature. Five ml. of 0.06M phosphate buffer, pH 6.35,\textsuperscript{8} was then added to each sample, and mixed by intermittent inversion of the test tube for 10 minutes. Each sample was then centrifuged, and the supernate (S\textsubscript{1}) decanted and saved. An additional 5 ml. of 0.06M phosphate buffer, pH 6.35 was added to each DEAE pellet. Each was then again mixed intermittently for 10 minutes and recentrifuged, decanting the supernate (S\textsubscript{2}) and saving both it and the remaining DEAE pellet.

Radioactivity was determined in the two buffer eluates (S\textsubscript{1} and S\textsubscript{2}) and in the DEAE pellets in a well-type scintillation counter.\textsuperscript{8} This was converted to picograms radioactive B\textsubscript{12} by comparison of the counting rate obtained with that of a radioactive B\textsubscript{12} standard. Total picograms of B\textsubscript{12}-binding globulins were determined, and percent α- and β-binding globulins were then determined by considering the two buffer eluates (S\textsubscript{1} and S\textsubscript{2}) as representing β B\textsubscript{12}-binding globulins, and the pellet as containing the B\textsubscript{12}-binding α-globulins.

Percent α and β B\textsubscript{12}-binding globulins obtained by this procedure were compared to percents obtained on identical serum samples separated by DEAE cellulose chromatography ("baby column" method) using the technic of Retief et al.\textsuperscript{8}

Cellulose acetate strip electrophoresis in barbital buffer, pH 8.6, was performed on both buffer eluates (S\textsubscript{1} and S\textsubscript{2}) from samples from normal and CML sera, to determine whether α-globulin contaminated these eluates, which should contain only β-globulin.

**Results**

Table 1 presents the results of ten sequentially run samples of serum tested via the two batch separation procedures and compared to results obtained using the "baby column" technic. The first nine samples were serum, while the last one was heparinized plasma. Our experience has been that the B\textsubscript{12} binders

\*Alternatively, radioactivity could have been determined in only eluates or pellet. How-ever, by counting both we correct for the approximately 15 percent of counts lost from the starting material in the various transfers of solution.
Table 1.—Comparison of percentage $\alpha$ and $\beta$ B$_{12}$ binders of 10 samples using batch vs. column separation

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Batch Separation Using washed DEAE Pellet</th>
<th>Batch Separation Using 0.170 gm. Dry DEAE</th>
<th>DEAE Column Chromatography using “Baby Column”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>% Beta 70.6 % Alpha 29.4</td>
<td>% Beta 72.9 % Alpha 27.1</td>
<td>% Beta 76.18 % Alpha 23.19</td>
</tr>
<tr>
<td>Normal</td>
<td>% Beta 72.1 % Alpha 27.9</td>
<td>% Beta 73.8 % Alpha 26.2</td>
<td>% Beta 78.02 % Alpha 21.98</td>
</tr>
<tr>
<td>Normal</td>
<td>% Beta 71.4 % Alpha 28.6</td>
<td>% Beta 70.4 % Alpha 29.6</td>
<td>% Beta 78.82 % Alpha 21.18</td>
</tr>
<tr>
<td>Normal</td>
<td>% Beta 70.7 % Alpha 29.3</td>
<td>% Beta 71.6 % Alpha 28.3</td>
<td>% Beta 75.5 % Alpha 24.5</td>
</tr>
<tr>
<td>Normal</td>
<td>% Beta 68.2 % Alpha 31.7</td>
<td>% Beta 73.0 % Alpha 27.0</td>
<td>% Beta 80.6 % Alpha 19.4</td>
</tr>
<tr>
<td>CML</td>
<td>% Beta 27.4 % Alpha 72.6</td>
<td>% Beta 28.7 % Alpha 71.3</td>
<td>% Beta 30.9 % Alpha 69.1</td>
</tr>
<tr>
<td>CML</td>
<td>% Beta 59.4 % Alpha 40.6</td>
<td>% Beta 60.8 % Alpha 39.2</td>
<td>% Beta 60.7 % Alpha 39.3</td>
</tr>
<tr>
<td>PV</td>
<td>% Beta 57.4 % Alpha 42.6</td>
<td>% Beta 63.0 % Alpha 37.0</td>
<td>% Beta 54.7 % Alpha 45.3</td>
</tr>
<tr>
<td>CML</td>
<td>% Beta 26.0 % Alpha 74.0</td>
<td>% Beta 28.1 % Alpha 71.8</td>
<td>% Beta 32.6 % Alpha 67.3</td>
</tr>
<tr>
<td>CML</td>
<td>% Beta 25.3 % Alpha 74.7</td>
<td>% Beta 31.7 % Alpha 68.3</td>
<td>% Beta 24.6 % Alpha 76.3</td>
</tr>
</tbody>
</table>

CML = chronic myelogenous leukemia.
PV = polycythemia vera.

of either serum or heparinized plasma may be successfully separated by the batch procedure.

Cellulose acetate strip electrophoresis demonstrated almost no $\alpha$-globulin in the first buffer eluate, and very small quantities in the second buffer eluate. Therefore, the phosphate buffer employed to elute only $\beta$-globulins successfully did so, removing little of the B$_{12}$ $\alpha$ binders.

**DISCUSSION**

The batch separation procedure described here uses the same concepts as the “baby column” chromatographic technic for separating B$_{12}$-binding proteins. Fahey et al. had determined that maximal elution of $\beta$-globulins took place at a molarity of 0.08 while a molarity of 0.15 was required to elute $\alpha$-globulins. Therefore, using a phosphate buffer of 0.06 M to elute the B$_{12}$-binding proteins allows the $\alpha$-globulins to remain in the pellet of DEAE. It is not necessary to elute these B$_{12}$-binding $\alpha$ binders, since their quantity can be determined directly by counting the radioactivity in the DEAE pellet; the radioactivity in the buffer eluates plus that in the pellet determines total B$_{12}$ binders.

The percent $\alpha$ and $\beta$ B$_{12}$-binding globulin as determined by the batch separation procedure is quite comparable to that obtained by column chromatography. Batch separation is easier for routine work than is column chromatography. All that is required is that the laboratory be equipped to handle radioactive isotopes. The procedure is simple; most of the time required is taken up by the centrifugations; the actual separation procedure requires little attention.

**SUMMARY**

A procedure has been described for the rapid and separate measurement of B$_{12}$-binding $\alpha$- and $\beta$-globulins using the batch technic. The method involves saturating B$_{12}$-binders with Co$^{57}$B$_{12}$, removing excess radioactive B$_{12}$ with a hemoglobin-coated charcoal pellet, and then separating the B$_{12}$ binders using
a pellet of DEAE cellulose, from which only the β-globulins are eluted in 0.06 M phosphate buffer at pH 6.35.

SUMMARIO IN INTERLINGUA

Es describite un procedimento pro le rapid e separate mesuration de globulinas α e β a capacitate B₁₂-ligatori con le uso del metodo "a lots." Iste metodo consiste in saturar ligatores de B₁₂ con Co⁵⁷B₁₂, remover le excesso de B₁₂ radioactive con le uso de un granulo de carbon de ligno a revestimento hemoglobinic, e separar subsequentemente le ligatores de B₁₂ con le uso de un granulo de cellulosa a DEAE ab le qual solo le globulinas β es eluite in un tampon a 0.06 phosphato con un pH de 6,35.

ACKNOWLEDGMENTS

We are indebted to Misses Le Teng Go, Melody Lee, and Leona Bandel for technical assistance.

Initial experiments suggesting the feasibility of batch separation of B₁₂ binders were carried out by Dr. Francois Retief in our laboratory during his tenure of U.S.P.H.S. International Postdoctoral Fellowship Award #F05TW 918.

REFERENCES

Brief Report: Rapid Determination of Vitamin B₁₂-Binding $\alpha$ and $\beta$ Globulin in Serum

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