Comparative Studies on the Microbiological Vitamin B12 Assay at Two Laboratories

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Received for publication 2 January 1976

The turbidimetric methods in routine use at two laboratories for the microbiological assay of vitamin B12 have been compared. Attempts were made to standardize some major parts of the method, i.e., assay design, test strain (Lactobacillus leichmannii), test medium, and reference standard. The laboratories used different approaches to achieve efficient assay procedures. During a 6-year period four comparative experiments were carried out. In these experiments the vitamin B12 content of five different products was determined in a series of independent assays at each laboratory. A satisfactory degree of agreement (difference <5%) was found for four of these products.

Microbiological turbidimetric vitamin B12 assays using Lactobacillus leichmannii as the test organism (4, 8, 9) have been carried out in the quality control laboratories of Philips-Duphar B. V. (Weesp, Holland) and Organon International B. V. (Oss, Holland) for more than two decades. At the beginning of this period many replicate assays had to be performed to obtain a satisfactory result. The main problems at that time were the lack of an appropriate vitamin B12 reference standard and a suitable test strain, as well as the maintenance of the test strain and the composition of the test medium. Since then, in both laboratories continuous efforts have been made independently to improve the accuracy and the reproducibility of the assay method (1).

First contacts between the two laboratories were established in 1967 when a large discrepancy (>10%) in vitamin B12 content was found in one product.

With the aim of decreasing this discrepancy a collaborative study of the assay method was undertaken.

The respective routine assay procedures were carefully re-evaluated and, where possible, standardized. During this period four comparative studies of the vitamin B12 content of a number of pharmaceutical products were carried out.

A description of the assay methods used at both laboratories and the results obtained are presented.

MATERIALS AND METHODS

The assay procedure for the turbidimetric determination of vitamin B12 (Fig. 1) consists of the following steps: (i) the preparation of concentrated solutions of samples and standards, (ii) the preparation of test cultures ready for incubation, (iii) the incubation period, (iv) the turbidity reading, and (v) the calculation of the assay results.

At Philips-Duphar the preparation of the test cultures included the preparation of the two dose levels, addition of test broth, autoclaving, and addition of inoculum. In the semiautomated method used at Organon (2) the test broth was inoculated; hence no autoclaving was carried out.

The methods used at both laboratories were standardized as far as possible with respect to the following. At both laboratories the assay designs were based on the two-point parallel line assay (3). The log dose-response curves yielded a steep slope within the range of 20 to 100 pg/ml. The ratio between the high and the low dose level was 2.

An identical isolate of L. leichmannii ATCC 7830 was used, taken from a stock stored in liquid nitrogen.

The stock culture was prepared by using either Dano vitamin B12 assay medium (Dano Chemo, Denmark), including 0.075 ng of vitamin B12 per ml, or AOAC lactobacilli broth (Difco) in culture tubes. The tubes were inoculated and were incubated for 16 to 18 h at 37°C. The cells were then harvested after three centrifugation and rinsing cycles, using 0.9% saline solution as rinsing fluid. The resulting sediment was resuspended to give a concentration of approximately 10^6 cells/ml. Portions of this suspension were divided over screw-cap bottles or ampoules, which were stored in the gas or liquid phase of liquid nitrogen, respectively. Once this stock culture was established, 0.2-ml aliquots of the thawed culture served as inoculum for a new "mother" culture. As a precaution, in addition to the above stock culture, a culture of the test strain was maintained on yeast extract-glucose-calcium-carbonate-agar (yeast extract, 10 g; glucose, 20 g; calcium-carbonate 10 g; agar [Difco], 15 g; and distilled water, 1,000 ml;
concentrated solutions

B preparation of test cultures
test cultures
C incubation
grown test cultures
D turbidity reading
extinction values
E calculation of assay results

Fig. 1. Flow diagram of assay procedure. Concentrated solution: Philips-Duphar, vitamin B12 concentration of approximately 4 ng/ml; Organon, vitamin B12 concentration of approximately 50 ng/ml.

the pH was adjusted to 6.4). This culture was stored at +4 or −26 C and transferred once a month. Highly purified cyanocobalamin (Glaxo) served as home reference at both laboratories. Twenty-two milligrams were carefully weighed on a semi-microbalance and dissolved in 25% ethanol to give a final concentration of approximately 20 μg/ml. The resulting solution was checked spectrophotometrically. When stored in a brown glass bottle at 4 C this solution could be used for periods up to 12 months. The commercially available Dano vitamin B12 assay medium was used as test medium.

Four-milliliter volumes of test culture in test tubes with an outside diameter of approximately 12 mm provided appropriate microaerobic conditions throughout the entire liquid column.

The turbidity was measured at wavelengths >600 nm and expressed in extinction values.

The extinction values, together with other data required for calculation of the vitamin content of the products, were transferred to paper tape or punch cards and were fed into a computer. The computer was programmed to calculate the vitamin B12 content of the samples, expressed in terms of the vitamin reference standard.

From 1969 onwards, four comparative studies were performed, i.e., in 1969, 1970, 1974, and 1975. The samples used in these studies were: vitamin B12 stock solution in 25% ethanol (home reference), U.S.P. Cyanocobalamin Reference Standard, Pernaemon Forte (Organon Int. B.V.), feed grade powder (Merck, Sharp & Dohme), and liver extract paste (Philips-Duphar B.V.).

The samples were assayed in duplicate according to the respective laboratory procedures described below. The results for the individual samples were obtained in a series of independent assays. In the first two studies the series included different numbers of assays, ranging from 3 to 10. In the latter two studies the series included 10 individual assay results. In the 1975 assay the Philips-Duphar extraction method was used at both laboratories for liver extract paste.

Philips-Duphar assay procedure. The assay was carried out in duplicate by two technicians on the same day.

(i) The concentrated solutions of the standard and samples, vitamin B12 concentration of approximately 4 ng/ml (Fig. 1), were prepared with distilled water, using Eppendorf micropipettes of 10 to 1,000 µl and volumetric flasks (50 to 250 ml).

All solutions were, as far as possible, stored in the dark. Each technician prepared one single dilution from the vitamin B12 home reference stock solution and one from the samples. A 50-mg portion of the U.S.P. Reference Standard powder was carefully weighed and transferred into a 50-ml volumetric flask with 25% ethanol. To the final dilution 2 μg of KCN per ml was added. One gram of feed grade powder was carefully weighed and transferred into a 100-ml beaker into which were added 25 ml of distilled water, 20 mg of KCN, and 25 ml of 0.2 M acetate buffer (pH 4.5), respectively. After heating for 15 min at 100 C the suspension was cooled in running tap water and further diluted. One gram of liver extract paste was carefully weighed and transferred into a 200-ml volumetric flask with distilled water. To the final dilution 2 μg of KCN per ml was added.

(ii) The resulting concentrated solutions were transferred into the culture tubes with Eppendorf pipettes of 10 and 20 µl. The tubes were placed in specially designed racks containing 30 holes. The two sets of four solutions (two low and two high dose levels) representing one sample were divided over two racks so that they were situated at different positions in the racks (Fig. 2). For each additional set of two samples a new rack was used, in which case the tubes containing the standard were moved to the next two positions in the rack.

The assay medium was prepared according to prescription by dissolving 73.3 g in 2,000 ml of distilled water. Four milliliters of this medium was transferred into each tube with a 5-ml Cornwall pipetting unit. All the tubes in the rack were covered with one stainless-steel cap and autoclaved for 5 min at 120 C.

Fig. 2. Position of sample tubes in the racks used at Philips-Duphar. Symbols: 0, blank (in the second and following racks position 1 is empty); O, low dose level; □, high dose level; ●, empty place.
After autoclaving and cooling the tubes were inoculated with approximately 0.05 ml of a diluted stock culture suspension of the test organism, containing approximately $10^2$ viable cells/ml.

(iii) The inoculated tubes in the racks were incubated at 37 C for 64 h in a thermostatically regulated incubator.

(iv) At the end of the incubation period the content of the tubes was homogenized and the turbidity of the cultures was measured in situ in the test tubes at a wavelength of 660 nm in a Unicam SP 400 spectrophotometer.

Birefringence of the rod-shaped L. leichmannii caused no problems during the measurement when the cultures were measured in the tubes between 1 and 10 min after homogenization.

The cuvette holder (see Fig. 3) consisted of a tube 20 mm in diameter, filled with water and closed by an ebonite top. The culture tube fit snugly into this tube through a hole in the ebonite top. In each assay the vitamin content was calculated from the extinction values representing one sample. The final assay result was obtained by calculating the mean of the two independent assay results.

**Organon assay procedure.** All procedures were carried out avoiding direct sunlight.

(i) Standard and sample solutions were diluted with distilled water to give a vitamin B12 concentration of approximately $50$ ng/ml (see Fig. 1) using volumetric pipettes (2 to 25 ml) and volumetric flasks (50 to 1,000 ml). Seven concentrated solutions were prepared from the vitamin B12 home reference stock solution, whereas the sample solutions were prepared in duplicate. A $50$ mg amount of the U.S. Cyanocobalamin Reference Standard was carefully weighed and transferred into a volumetric flask and dissolved in a 25% ethanol solution. Two milliliters of a $0.1\%$ KCN solution was added to 100 ml of the final solution. A 2.5-ml portion of Pernaemon Forte was pipetted into a volumetric flask containing 2 ml of a $0.1\%$ KCN solution and further diluted. Approximately $1$ g of feed grade powder was weighed and transferred into a 150-ml beaker. Two milliliters of a $0.1\%$ KCN solution and 20 ml of distilled water were added, and the suspension was treated with a sonifier (Branson) for 1 min at 70 W. The suspension was then diluted further. Approximately $5$ g ($\pm 5\%$) of liver extract paste was weighed in a 100-ml beaker. A $10$ ml amount of distilled water of $80$ C was added. After careful stirring, the solution was cooled and transferred into a volumetric flask containing 2 ml of a $0.1\%$ KCN solution. The resulting solution was diluted further.

(ii) Each concentrated solution representing one individually prepared standard or sample was divided over two sample tubes. The sets of two tubes forming the experimental unit were then placed in random order in the racks of an automatic dilution and dosage apparatus. As described elsewhere (2), this apparatus prepared a high and low dose test culture from each concentrated solution.

Hence, each concentrated solution yielded four test cultures (two high and two low dose levels). The random order was further maintained throughout the entire assay procedure, including the turbidity reading. The inoculated test broth was prepared as follows. To obtain a short lag phase, the test organism taken from the liquid nitrogen was precultured. For this purpose $10$ ml of AOAC lactobacilli broth (Difco) in a culture tube was inoculated with 1 ml of the thawed stock culture. After incubation at $37$ C for $16$ h the culture was centrifuged at $3,000$ rpm for $10$ min, and the sediment was rinsed with a sterile physiological saline solution. This procedure was repeated twice, and the resulting sediment was dissolved in $10$ ml of saline solution and homogenized. To ensure rapid growth, concentrated Dano assay medium ($73.3$ g/750 ml) was used. The Dano assay medium was inoculated by adding $0.3$ ml of the above homogeneous inoculum suspension (viable count, approximately $10^7$ cells/ml) for each 100 ml.

(iii) The inoculated tubes in the racks were covered with stainless-steel caps and stored in cold water until all tubes included in one assay had been filled.

The culture tubes in the racks were then incubated at $37$ C for $16$ to $18$ h in a thermostatically controlled water bath.

(iv) After incubation the growth was stopped by immersing the racks in cold water. The bacterial suspensions were homogenized, and the turbidity was measured automatically at a wavelength of $653$ nm (2).

A vitamin content was calculated from each set of four turbidity values resulting from each concentrated solution. The mean of the contents of the duplicates determined in one assay yielded the final assay result for each sample.

**RESULTS**

The results of the comparative studies performed at both laboratories in 1969, 1970, 1974, and 1975 are summarized in Table 1. The vitamin contents given are the geometric means of the individual assay results obtained in a series of assays.

The following differences in vitamin B12 content were found: U.S.P. Reference Standard, 5, 4, and 3% in 1969, 1970, and 1974; home refer-
ence, 2% in both 1970 and 1974; Pernaemon Forte, 2 and 0% in 1969 and 1974; feed grade, 2 and 0.5% in 1970 and 1975; liver extract paste, 5, 7, 16, and 3% in 1969, 1970, 1974, and 1975, respectively.

In Table 2 all the individual assay results, obtained at the respective laboratories in the 1975 comparative study, are presented. For Philips-Duphar, the first two columns represent the series of duplicate contents as determined by two technicians on 10 different days. For Organon, the duplicates were obtained in one assay. The vitamin B12 contents obtained for liver extract paste at Philips-Duphar and Organon ranged from 9.8 to 11.5 and 9.5 to 11.2 \( \mu g/g \), respectively, and for feed grade powder they ranged from 454 to 566 and 468 to 521 \( \mu g/g \), respectively.

**DISCUSSION**

The complex nature of a microbiological vitamin assay implies that many differences will remain between the execution of the assay at various laboratories. These differences are due to the fact that each microbiological laboratory has its own "traditions" with respect to work procedures, glassware, instruments, etc. Additionally, the work routine at each laboratory varies with the room, the number of assays to be performed, etc.

As is shown in the description of the assay procedure, we had some success in standardizing the assay design, test strain, test medium, and reference standard.

From the start of the routine performance of vitamin B12 assays at both laboratories much attention was paid to finding the most efficient statistical design. The two-point parallel line assay proved to be the method of choice.

Daily transfer of *L. leichmannii* is a laborious procedure. In addition, changes in the characteristics of the test strain may occur, and the chance of contamination and inconsistency of media exist. Inexplicable changes occur rather frequently when using a test strain that is transferred frequently. These changes, causing many assay failures, have been completely eliminated since stock cultures stored in liquid nitrogen have been used (7). The introduction of liquid nitrogen for preserving the inoculum has the additional advantage that inocula originating from one identical batch of the test organism can be used for long periods. In this way variation in the inocula for assays performed on different days can be reduced to a minimum. Experience at Philips-Duphar since 1969 with such cultures has shown that they are stable for at least 1 year. The preparation of the complex test broths used for vitamin assays involves risks of introducing inconsistencies that are often hard to trace. For this purpose well-standardized, commercially available assay media may provide a good substitute. For vitamin B12 the Dano medium was found to be far more reliable than other commercially available test broths. Only negligible differences in growth response were found between various batches of this Dano medium in routine use during the
last 8 years. Accurate preparations of the standard is of vital importance. Highly purified and spectrophotometrically checked cyanocobalamin or U.S.P. Reference Standard can satisfactorily serve as standard. The tedious procedure of weighing, drying, and dissolving the vitamin B12 reference standard can be replaced by using a stock solution that can be used for longer periods. Studies performed at the laboratory of Philips-Duphar revealed that cyanocobalamin solution in 25% ethanol proved to be stable for periods of up to 1 year when stored at 4°C.

It should be realized that, even after standardization, important differences between the procedures still remain. At Philips-Duphar the contents of the products are determined by two technicians each in one assay on the same day, whereas at Organon they are determined in duplicate in one assay. In the first case each technician carries out the entire procedure. At Organon a team consisting of two to four technicians carries out all manipulations involved. The use of the automated method at Organon means that the diluted samples are mixed with inoculated test medium and dispensed into tubes without any heating.

At Philips-Duphar the tubes with the test mixtures are autoclaved prior to inoculation. At Organon, in addition, a relatively short incubation period of 16 h is used and the dose-response curve obtained gives the relation between vitamin content and growth rate. In the case of Philips-Duphar a 64-h incubation period is used, and here the total cell yield is a function of the vitamin content.

In turbidimetric assays the growth is usually measured by using either spectrophotometers or colorimeters. These instruments are intrinsically unsuited to this purpose, because turbidity is measured in terms of extinction values. Experiments performed by both laboratories show that, indeed, each colorimeter has its own reading characteristics, which emphasizes the need for stable, reliable instruments. At the two collaborating laboratories, different methods were used which both yielded acceptable results. The wavelength chosen was >600 nm, since at lower wavelengths the extinction values are disturbed by metabolites produced by the bacterial culture.

At the start of the collaborative study, the assay procedure at each laboratory had been in use for more than 20 years, and each gave satisfactory results. During this study modifications of the laboratory procedures have been introduced which, in the case of Organon, have resulted in an increased reproducibility and a reduction in the occurrence of assay failures (1). The improvement should be attributed to the improved standardization of inocula. At
Philips-Duphar, a more efficient and accurate procedure for data transmission into the computer was developed by using paper tape instead of punch cards.

Both assay procedures provide different solutions to the problem of the workload involved in microbiological assays. In the case of a relatively small number of samples, the Philips-Duphar method, dilution with Eppendorf pipettes, provides a cheap and efficient method. The semiautomated method used at Organon proved its value in assaying great numbers of samples, avoiding most of the monotonous manual work. The methods used for turbidity reading at both laboratories, e.g., in situ per tube and in a flow cell, proved to be efficient in the routine of each individual laboratory with their differences in workload. Finally, the computerization of the calculation eliminates much laborious work. At Philips-Duphar two technicians can easily assay 180 samples per week. At Organon, 600 samples are routinely analyzed by five technicians using three diluting and dosage units and one turbidity reading unit.

In the comparative experiments carried out during the last 6 years only in the case of liver extract paste were relatively large discrepancies found. In the four experiments, differences between the two laboratories ranged from 3 to 16%. To find out whether the remaining differences between the methods had caused the discrepancies for this particular product, some possible contributory factors were studied, e.g., extraction method, heat treatment of the sample, test medium mixture, and the length of incubation period. None of these factors could be shown to contribute to the observed discrepancies. Other possible contributory factors are being investigated. For the other products, U.S.P. vitamin B12 Reference Standard powder, the home reference B12 solution, Pernaeum Forte solution, and feed grade powder, good to excellent agreement (0.5 to 5%) was obtained between the vitamin B12 contents determined at both laboratories. The low day-to-day variation obtained at both laboratories demonstrates the good reproducibility of the assay procedures.

ACKNOWLEDGEMENT

We are grateful to H. A. Behagel and R. van Strik for their advice, and to J. Enserink, Grada van Kesteren, Rosanna Jongbloets, Adelaine Schoolderman, and Annie Manders for their assistance.

LITERATURE CITED